PANCREATIC ISLET TRANSPLANTATION IN THE MOUSE

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STATEMENT

The experiments in Chapter Six were carried out in collaboration with Dr. E. Parr, and those in Chapter Seven in collaboration with Ms. C. Simeonovic. With these exceptions, the experiments documented in this thesis were performed by myself.

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ABSTRACT

The culture of BALB/c mouse pancreatic islets in a gas phase of 95% oxygen for 4-7 days prior to their transplantation across major histocompatibility barriers was shown to either abolish or markedly reduce their immunogenicity. Cultured BALB/c islets allografted to normal non-immunosuppressed CBA recipients survived completely intact for at least 420 days following transplantation, and the allotransplantation of 350 cultured BALB/c islets under the renal capsules of streptozotocin-diabetic CBA mice were shown to promptly reverse their diabetes.

A comparative histological study showed that BALB/c foetal pancreas required a 17 day culture period before loss of immunogenicity became apparent. Lymphoid elements of apparent donor origin were seen in association with BALB/c islet isografts, and the prolonged period of organ culture required prior to allografting may have been necessary to completely destroy donor-strain lymphoid tissue.

Cultured allografts of BALB/c islets grafted to normal non-immunosuppressed CBA mice were readily rejected following

challenge of the host animals with donor-strain peritoneal leucocytes either at the time of grafting, or at a year following transplantation, which showed that the cultured islets had retained transplantation antigen and was consistent with the hypothesis that organ culture under conditions of high oxygen tension reduces tissue immunogenicity by destroying stimulator cells prior to transplantation. In contrast, 50% of those CBA animals receiving 300-350 BALB/c islets following induction of diabetes with streptozotocin subsequently failed to reject their allografts after repeated challenge with donor-strain peritoneal cells. Evidence is also presented showing that a non-specific stimulus to the host immune system is not likely to bring about islet allograft rejection in mice made diabetic prior to allotransplantation.

An ultrastructural study of islets and thyroid lobes demonstrated that organ culture led to loss of vascular endothelium, and also substantiated the evidence that cultured thyroid tissue continues to express transplantation antigens.

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CHAPTER ONE

GENERAL INTRODUCTION

CHAPTER ONE

INTRODUCTION

In 1921 the purification of bovine insulin by Banting and Best revolutionised the medical treatment of the juvenile onset form of diabetes mellitus. This type of diabetes is characterised by a deficiency in endogenous insulin production, and prior to the introduction of insulin therapy, sufferers would generally succumb to the disease within weeks or months of its onset. Insulin enables most of these diabetics to live near normal lives for many years, but unfortunately it does not effect a cure, and many insulin dependent diabetics are at risk from the increased morbidity and mortality that is associated with a now chronic disease state. Conventional insulin treatment rarely restores the 24 hour blood glucose profile to normal, and available evidence suggests that many of the long term complications of the disease may be due to pathological changes induced in the microvascular bed by chronic intermittent hyperglycaemia. Additionally, not all diabetics stand to benefit from insulin therapy, for although diabetes mellitus is broadly defined as a disease state characterised by glucose intolerance, there are three major

disease sub-types, each of which is mutually exclusive. (National Diabetes Data Group 1979).

Type I or insulin dependent diabetes mellitus (IDDM) is seen principally in persons under the age of 30 years at onset, and is characterised by acute development of the symptoms and signs of hypoinsulinaemia and ketosis. Insulin administration is essential, otherwise death will eventually ensue. The cause of the disease is unknown; an increased frequency is seen in association with certain histocompatibility antigens in chromosome 6, chiefly B8, B15 and D/DrW3 and 4 (Rotter & Rimoin 1978; Thomsen *et al* 1979) indicating a genetic pre-disposition to the disease. A number of viral infections, in particular mumps, rubella and coxsackie B4, have also been associated with onset of IDDM, but an infectious aetiology (Yoon *et al* 1979) is rarely proven. Islet cell antibodies are associated with onset of the disease in some instances, but whether they are a cause or effect phenomenon is still unclear. They may appear in response to islet cell damage due to viral infection (Lendrum & Walker 1975) or reflect an ongoing autoimmune disease progress (Bottazzo, Florin-Christensen & Doniach 1974).

Type II or non insulin dependent diabetes mellitus (NIDDM) is more common than the insulin dependent type of diabetes and usually occurs after the age of forty years. It is often associated with obesity. Insulin is not usually indi-

cated as first line therapy in the early stages of Type II diabetes, as oral hypoglycaemic drugs plus a suitable diet will often alleviate many of the acute symptoms of hyperglycaemia.

Diabetes mellitus may also occur in individuals who are suffering from a separate disease state which in turn generates secondary glucose intolerance. This form of diabetes may be associated with generalised pancreatic disease, hormonal disturbances that produce either secondary hypoinsulinaemia or insulin resistance, drug induced diabetes, insulin receptor abnormalities, and numerous genetic syndromes (National Diabetes Data Group 1979).

It is apparent that the treatment of diabetes mellitus by various combinations of insulin, diet, and oral hypoglycaemic drug therapy is not sufficient to prevent long term complications of the disease from developing in some diabetics. Sufferers from IDDM are particularly prone to developing a micro-angiopathy that can involve the retina, kidneys, peripheral nervous system, skin and the organs innervated by the autonomic nervous system. In Western countries, diabetes rates as a common cause of blindness and diabetic nephropathy, and is responsible for an increased incidence of morbidity and premature death amongst the diabetic population (Deckert *et al* 1978; Bale & Entmacher 1977). As well, the peripheral and autonomic neuropathies suffered by a significant proportion of diabetics

are often extremely uncomfortable, and at times are incapacitating.

The cause or causes of diabetic microangiopathy are not well understood. It has been demonstrated that increase in capillary basement membrane thickening is a function of the duration of diabetes (Williamson & Kilo 1977) and it has been postulated that the increased deposition and subsequent trapping of sorbital, a by-product of glucose metabolism, within basement membranes is a major cause of membrane damage (Heath & Hamlett 1976). Other factors, such as coagulation disturbances, increased vascular permeability and localised tissue hypoxia may also be involved, particularly in the pathogenesis of diabetic retinopathy where capillary leakage, vascular proliferation in response to tissue hypoxia, and fibrosis may co-exist in severe cases (Palmberg 1977).

Animal studies in inbred strains have clearly shown that reversal of streptozotocin or alloxan induced diabetes can be achieved by isotransplantation of pancreatic islets. Animals rendered diabetic by streptozotocin or alloxan develop the characteristic microvascular lesions of the disease within a few weeks of its induction, and reversal of the chemical diabetes with islet isografts also leads to the gradual disappearance of the vascular lesions in the eye and kidney (Mauer *et al* 1974; Krupin *et al* 1979). Although it has been difficult to prove that good control

of hyperglycaemia lessens the development of complications in human diabetes (Tchobroutsky 1978) these results suggest that the long term correction of abnormal glucose tolerance in diabetics might favourably influence the progression of vascular complications. Unfortunately, the usual treatment of IDDM with daily or twice daily subcutaneous administration of porcine or bovine insulin rarely returns glucose tolerance to normal (Service *et al* 1970) although there are data suggesting that progression of mild diabetic retinopathy can be slowed by means of two or three insulin injections a day (Job D. *et al* 1976; Eschwege *et al* 1979; Waltman *et al* 1979).

Continuous infusion of insulin, via the subcutaneous or intravenous route, has recently been shown to significantly improve long term glucose tolerance in insulin dependent diabetics (Pickup *et al* 1979; Irsigler & Kritz 1979) and a recent case report has indicated that continuous insulin administration may reverse the progression of serious diabetic retinopathy (Irsigler *et al* 1979). The infusion apparatus is relatively bulky at present, but small implantable pumps that could solve this problem are under development (Blackshear *et al* 1979). Attempts to bypass parenteral insulin administration have been made using insulin incorporated into gut-absorbable liposomes but trials in human volunteers have not been particularly encouraging (Patel *et al* 1978). Recently, a novel method

for mimicing the physiological control of insulin release has been proposed. It involves the release of free insulin from a lectin-insulin depot complex in quantities proportional to ambient glucose concentration (Brownlee & Cerami 1979). If the system works *in vivo*, it could be beneficial to diabetics by improving overall glucose tolerance and at the same time avoiding some of the more vagarious side-effects of present insulin therapy.

At present, the successful allotransplantation of endocrine pancreas into insulin dependent diabetics probably offers the best solution to the long term complications of diabetes, for such grafts have several potential advantages over other means of insulin delivery. These include precise and physiological control of insulin release in response to carbohydrate intake, a potentially inexhaustible supply of insulin and avoidance of the type of insulin resistance associated with the formation of anti-insulin antibodies to xenogeneic insulins (Burrows *et al* 1957). As experimental diabetes in animals produces similar complications to those seen in humans, and since it is clear that reversal of the diabetes by both isotransplantation and allotransplantation of endocrine pancreas will either prevent or cause regression of some of these lesions (Mauer *et al* 1974 ; Gray & Watkins, 1976; Krupin *et al* 1979), it is reasonable to hope for similar success with the treatment of human diabetes by organ transplantation, provided

graft rejection can be avoided.

Renal transplantation has already been shown to be a life-saving treatment for chronic renal failure due to diabetic nephropathy, (Simmons *et al* 1978) despite the need for adjunctive immunosuppressive therapy, with its capacity to kindle serious or even fatal bacterial, fungal and viral infections (Hall *et al* 1976; Toussaint *et al* 1978), and the association of immunosuppressive therapy with an increased risk of malignancy (Penn 1978). Nevertheless, the use of such therapy for the maintenance of functional renal allografts in diabetic patients provides justification for the subsequent experimental transplantation of endocrine pancreas to these individuals, the aim being the improvement of glucose tolerance, and the reversal or amelioration of the other co-existing complications of long-standing diabetes. If these desirable results could be obtained without increasing overall morbidity or mortality figures, then endocrine pancreas allografts could be offered to those diabetics who are on the verge of developing serious complications.

Assuming that such functional allografts will arrest the progress of the disease in man, this approach would largely circumvent the need for subsequent renal transplantation for diabetic nephropathy, save diabetics with retinopathy from ultimate blindness and conceivably retard the progression

of atheromatous vascular disease and the various diabetic neuropathies. Since there are grounds for believing that a cure of insulin dependent diabetes mellitus would lead to a better overall prognosis for the at-risk diabetic, such expectations demand an extensive research program directed towards solving the difficulties that have so far prevented the adoption of endocrine pancreas transplantation as the ultimate cure for insulin dependent diabetes mellitus.

Although data obtained from isograft experiments in rodents show that endocrine pancreas transplantation offers a potential cure for insulin dependent diabetes, the problem of allograft rejection has not yet been resolved, and until recently it has been difficult to achieve a consistent or significant prolongation of allograft survival time in animals. This is despite the concurrent use of various forms of immuno-suppressive therapy.

The results obtained by a number of investigations (Reckard & Barker 1973; Naji *et al* 1975; Nelken *et al* 1976; Finch & Morris 1976), imply that pancreatic islets are highly immunogenic, with allografts across major histocompatibility barriers in the rat generally undergoing functional rejection within 3 days of transplantation. In contrast, skin, heart, or kidney allografts between these same strain combinations took between 7 and 9 days to reject.

Attempts to prolong islet allograft survival by the treatment of recipient animals with enhancing serum (Finch & Morris 1976; Nash *et al* 1978), cyclophosphamide (Finch & Morris 1977), steroids (Nelken *et al* 1976), azothioprine (Marquet & Heystek 1975), cyclosporin (Vialettes *et al* 1979; Garvey *et al* 1979; Bell *et al* 1980) or antilymphocyte serum (Marquet & Heystek 1975; Nelken *et al* 1976; Gray & Watkins 1976; Finch & Morris 1977), have been generally disappointing. These studies have failed to extend allograft survival for more than 100 days when donor and recipient rat strains differed at the major histocompatibility complex (MHC). Gray & Watkins produced consistent functional graft survival for at least 270 days with intermittent ALS therapy, but the rat strains used (Wistar-Lewis to Fischer) only differed at minor histocompatibility loci.

Treatment of the recipient strain with intraperitoneal silica both before and after transplantation of F1 hybrid islets will certainly prolong graft survival beyond 100 days (Nash *et al* 1980). Silica suppresses macrophage function, and presumably this therapy inhibits macrophage processing of islet antigen, but it is unlikely that this form of therapy will have practical application.

The results of human studies have been just as disappointing, as the majority of attempts to successfully allograft whole

pancreas, segmental pancreas, foetal pancreas or collagenase-digested pancreatic fragments to diabetic recipients have failed, with grafts rarely functioning for more than several months (Raskin 1979). Most of these failures have been attributed to functional failure of the graft secondary to either assumed or proven graft rejection, with the remainder due to post surgical complications or severe infection necessitating graft removal.

In the early studies no attempt was usually made to match the histocompatibility antigen haplotypes of donor and recipient, but one of the more recent series has had the donor and recipient matched for at least two antigens of the HLA-A and HLA-B loci (Sutherland *et al* 1980), whilst HLA-DR matching has also been attempted (Groth *et al* 1980). Unfortunately, no improvement over the earlier dismal results has yet been seen, and many more allografts will need to be performed before any benefit of such matching becomes apparent.

Nevertheless, since the occasional pancreatic allograft will function indefinitely (Raskin 1979; Largiader, Kolb & Binswanger 1980), with production of sufficient endogenous insulin to enable either partial or complete cessation of parenteral therapy, it is evident that provided there are no surgical complications or serious infection following transplantation, the major barrier to successful allografting of endocrine pancreas is graft rejection.

The phenomenon of allograft rejection was first studied systematically about 60 years ago, when genetically identical inbred strains of mice started to become available. Empirical observations made in the late nineteenth and early twentieth century by such workers as Loeb & Little suggested that graft rejection was related to genetic differences between the donor and recipient (Little 1914; Loeb 1945). This correlation was further clarified by Peter Gorer in the mid-thirties, when he showed that certain inbred mouse strain tumours carried genetically determined tumour antigens that were associated with either acceptance or rejection of the grafted tumour by the host (Gorer, 1937). Gorer termed these antigens H, (histocompatibility) antigens, and it has since been shown that nearly all classes of mammalian cells carry such antigens on their surface membranes.

Medawar expanded the concept of antigen initiated graft rejection when he examined the kinetics of skin allograft rejection, and demonstrated that graft rejection was accelerated if the recipient animal had received prior exposure to donor-strain tissue antigens in the form of a previous skin graft (Medawar 1944). Accelerated graft rejection subsequent to a second challenge with allo antigen implied that the initial exposure had sensitised the animal to that antigen, and in a series of passive transfer experiments, Billingham, Brent & Medawar (1955) showed that the lymphocytes

or lymph node cells of a previously sensitised animal were capable of inducing accelerated (secondset) graft rejection in naive recipients of skin grafts. A year beforehand, Mitchison (1954) had demonstrated that the ability to raise an allograft reaction against tumour implants could be transferred from one animal to another by injecting a suspension of immune lymphocytes, and together, these findings illustrated the importance of the cellular immune response in allograft rejection. Billingham, Brent & Medawar (1956) also noted that living lymphocytes were 5 - 10 times more effective than tissue antigen preparations in inducing allograft rejection but they chose to ignore these observations, maintaining that allogeneic cells had no role in the genesis of graft rejection apart from providing a source of antigen.

In 1957, Snell suggested that leucocytes contained within a graft at the time of allotransplantation might be involved in the initiation of graft rejection. Subsequently, a number of investigators produced experimental evidence suggesting that passenger leucocytes contributed to graft immunogenicity (Elkins & Guttman 1968; Billingham 1971), but although the efficiency of transplantation antigen presentation via leucocytes was recognised, the reasons for this were not understood (Billingham, 1971). However, the clinical implications of any reduction of graft immunogenicity were obvious and many attempts were made to remove passenger leucocytes from organs such as the kidney. Methods used

included graft irradiation, donor pre-treatment with anti-lymphocyte serum, or flushing of the allograft with saline prior to transplantation. Unfortunately, most of these treatments did not consistently reduce graft immunogenicity (Billingham 1971). Nevertheless, donor pre-treatment with cyclophosphamide was shown to prolong the mean survival time (MST) of renal allografts in rats; presumably it reduced the number of passenger leucocytes within the graft at the time of transplantation (Guttman *et al* 1967). The nature of the *in vivo* interactions of allogeneic leucocyte populations was further examined by Lafferty & Jones (1969). In their studies of the graft versus host (GVH) and host versus graft phenomena, they demonstrated that interactions between allogeneic leucocytes were not necessarily stimulated by antigenic differences alone, and they concluded that the triggering of the immune response in an animal exposed to allogeneic cells of lymphoreticular origin might be mediated by a separate activation mechanism. This they termed allogeneic reactivity.

Bretscher & Cohn (1970) proposed that antigen presented to a responder B cell population will induce tolerance if a second inductive signal was not provided by antigen simultaneously binding to a carrier antibody that was capable of activating the responder cell, and it was subsequently suggested by Lafferty and his colleagues (Lafferty *et al* 1972; Lafferty & Cunningham 1975) that the activation of

allogeneic T lymphocytes may also depend on a 2 signal mechanism, provided by both major histocompatibility antigen differences (signal 1) and a second unspecified interaction (signal 2) between allogeneic lymphocytes. Such interactions can be studied *in vitro*, using the mixed lymphocyte reaction (MLR). In a unidirectional MLR where one of the two cell populations is metabolically inactivated by the use of irradiation, chemical inhibitors such as iodoacetic acid, or heat, no stimulation of the responder cell population is seen, despite the fact that histocompatibility antigens on the irradiated cell population remain intact (Shellekens & Eijsvoogel 1970; Lindahl-Keissling & Safwenberg 1971; Lafferty, Misko & Cooley 1974). These findings led to development of the concept that histocompatibility (transplantation) antigens act as strong immunogens only when they are presented on the surface of metabolically active immunocompetent cells, (Hardy & Ling 1969; Lafferty *et al* 1972; Lafferty, Misko & Cooley 1974) and provide a likely explanation for the importance of passenger leucocytes in sensitising the host to allografted tissues (Lafferty & Talmage 1976). Recent work by Batchelor, Welsh & Burgos (1978) has verified the concept that histocompatibility antigens have a poor intrinsic immunogenicity. They have shown that histocompatibility antigen presented on living spleen cells is 1000 times more potent in inducing an immune response *in vivo* than is the same amount of antigen

presented in pure form. Accordingly, the inactivation of leucocytes carried in an allograft prior to transplantation would presumably lead to a reduction in graft immunogenicity due to loss of stimulator leucocytes, but an additional benefit would be the removal of the responder population of leucocytes responsible for a non-specific and potentially destructive inflammatory reaction within the graft following stimulation by host type stimulator leucocytes (Elkins & Guttman 1968; Lafferty & Jones 1969; Lafferty & Talmage 1976; Lafferty *et al* 1976a).

In 1973, Summerlin *et al* presented evidence suggesting that organ culture of murine skin would prolong its survival following allotransplantation, and attributed this reduction in graft immunogenicity to loss of passenger leucocytes from the skin during the period of organ culture. Unfortunately, it was alleged that Summerlin had faked some of the results, and when other workers, including those from his own Institute (Ninnemann and Good 1974) were unable to reproduce his results, the idea that organ culture could favourably condition tissue for allotransplantation was temporarily discredited. However, following earlier observations that organ culture of certain mouse tumour lines enhanced their subsequent survival in allogeneic hosts (Jacobs & Huseby 1967), Jacobs (1974) was able to demonstrate that tissue culture could reduce the immunogenicity of tissue grafted across a major histocompatibility barrier. She showed that the culture of BALB/c

($H\text{-}2^d$) ovaries in 5% CO_2 and air for 6 - 12 days prior to transplantation into DBA/1($H\text{-}2^q$) recipients led to a significant increase over the control group in the number of allografts surviving and functioning 8 weeks after transplantation. Her results were soon confirmed by Leuker & Sharpton (1974).

Although Jacobs alluded to the possibility of passenger leucocytes being reduced in number by the culturing procedure, Lafferty was able to subsequently demonstrate that passenger leucocytes are likely to play a key role in the determination of allograft immunogenicity. He showed that the organ culture of mouse thyroid in a high oxygen gas phase for 28 days abolished their immunogenicity when grafted across a MHC barrier in the mouse. A high partial pressure of oxygen not only facilitates survival of parenchymal thyroid tissue during an extended period of culture (Lafferty *et al* 1975), but is probably also selectively toxic towards passenger leucocytes (Talmage & Dart 1978). Superoxide dismutase normally catalyses the toxic superoxide radical to hydrogen peroxide and oxygen, and it appears that incomplete catalysis of this radical under conditions of high (95%) oxygen tension leads to adverse effects on leucocyte metabolism (Rister & Baehner 1976). These include complete inhibition of lymphocyte DNA synthesis in the presence of mitogen (Andersen, Hellung-Larsen & Sorensen 1968; Lindahl-Kiessling & Karlberg 1979) and inactivation of the enzymes involved in the inter-

mediate metabolism of poly-morphonuclear cells and macrophages (Rister & Baehner 1976). Thus, prolonged organ culture under these conditions is likely to bring about either metabolic immobilisation or death of passenger leucocytes, and with this form of donor pre-treatment, thyroid allografts will survive completely intact for over 400 days (Lafferty 1980). Similar beneficial effects due to culture of rat parathyroids in a 95% O_2 atmosphere prior to transplantation have recently been demonstrated (Naji *et al* 1979). In the thyroid allograft studies the host animals were shown to be responsive to donor-strain antigens, as spleen cell preparations of animals bearing functional thyroid allografts could generate an *in vitro* cytotoxic T cell response to donor-strain spleen cells, and thyroid allografts underwent rejection if peritoneal leucocytes collected from the donor-strain were injected intravenously at the time of transplantation (Lafferty *et al* 1976b). However, attempts to reject these allografts using killed donor type peritoneal cells were not successful (Lafferty *et al* 1976c).

These experiments did not allow precise identification of the cell type(s) responsible for stimulating a host allograft response, but they do show that a cultured thyroid allograft still carries recognisable transplantation antigens, despite the loss of graft immunogenicity, and that living peritoneal cells injected at the time of transplantation

restore the host's ability to mount an immunological response to transplantation antigen, with subsequent rejection of the cultured allograft. Clearly these metabolically active cells are more than just mere hawkers of inert transplantation antigen. *In vitro* experiments have shown that allogeneic cytotoxic T cell precursors will not respond to UV irradiated stimulator cells, but the response may be restored by adding a lymphokine preparation obtained from concanavalin-A activated spleen cells (Lafferty *et al* 1978). Since UV irradiation has no detectable effect on cell surface histocompatibility antigens (Lindahl-Kiessling & Safwenberg 1971; Lafferty *et al* 1978), its action may be via suppression of endogenous lymphokine release. Nevertheless, activity of non-specific lymphokine preparations *in vivo* has yet to be convincingly shown (Kindred, Bosing-Schneider & Corley 1979). However, the finding that cultured thyroid allografts are only rejected when living donor-syngeneic peritoneal cells are injected into the recipient (Talmage *et al* 1976) is consistent with a 2 signal operational requirement for the induction of graft rejection (Lafferty & Cunningham 1975; Lafferty & Woolnough 1977).

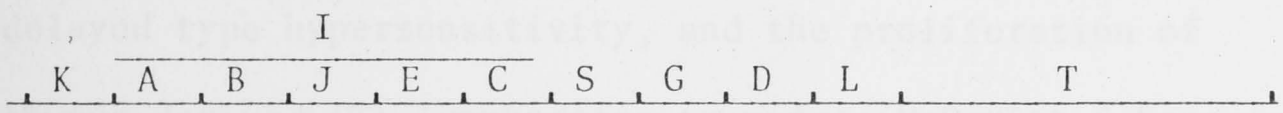
The cultured thyroid allograft model has proven to be extremely useful in expanding knowledge of the mechanisms of organ graft rejection, but there are few clinical indications for thyroid allotransplantation, as human thyroid

hormone deficiency states are readily corrected provided the patient complies with the thyroxine replacement therapy. Nevertheless, there are a number of hormone deficiency states that are not so easily corrected. These include hypoparathyroidism, hypopituitarism, primary hypogonadism, and insulin-dependent diabetes mellitus. In these conditions, hormone replacement therapy does not necessarily provide complete restoration of normal physiological function and may, in fact, produce adverse side-effects due to difficulty in optimizing drug dosage. The disease state providing the most common and most vivid example of these shortcomings is insulin-dependent diabetes mellitus. Since it is evident that grafting of endocrine pancreas will reverse both the metabolic abnormalities and microvascular complications associated with experimental diabetes in rodents, and that the major barrier to successful allotransplantation in mammalian species is graft immunogenicity, it is worthwhile attempting to reduce the immunogenicity of endocrine pancreas by using tissue culture. Accordingly, this thesis consists of a series of experiments designed to study the effects of organ culture in 95% oxygen on the immunogenicity and function of mouse pancreatic islets grafted across minor and major histocompatibility barriers.

In the mouse, the H-2 antigen system has been found to be linked with the most rapid onset of allograft rejection, (Snell 1957) and the genetic loci coding for these antigens

have been termed the major histocompatibility complex (MHC) of the mouse. This system has been extensively reviewed by Klein (1979). A brief summary is presented here.

The H-2 complex is located in the mid portion of mouse chromosome 17, and consists of 7 regions termed K, I, S, G, D, L, and T, with the I region sub-divided into 5 sub-regions.



Each region contains one or more loci, some of which may be identical, and according to their phenotypic expression, the loci are designated as Class I, II, or III (Klein 1979). Class I (H-2D, H-2K) loci code for membrane-bound glycoproteins with a molecular weight of 44,000 Daltons, and these are co-valently associated with beta$_2$ microglobulin within the cell membrane. Mitchison (1954) showed that lymphocytes are the prime mediators of allograft rejection, and it has been since shown using *in vitro* techniques, that it is possible to gene-rate cytotoxic lymphocytes directed against cells carrying H-2D or H-2K gene products (Alter *et al* 1973; Bach *et al* 1977).

Class II genes are located within the I region of the H-2 complex. There are two sub-classes; Ia and Ir. Ia genes code for membrane-bound glycoproteins that consist of two noncovalently linked proteins (alpha : 35,000 Daltons, and beta : 28,000 Daltons) and an unknown number of carbohydrate

chains (Cullen, Freed & Nathenson 1976). Ia gene products are thought to be responsible for the activation of lymphocytes in mixed leucocyte reactions (Meo *et al* 1975). I region differences may also lead to rapid rejection of skin allografts, while cell mediated lymphocytotoxicity reactions *in vitro* can also be mediated by such differences (Klein 1978). Immune response (Ir) genes located within the I region influence the humoral response to antigen, the generation of delayed type hypersensitivity, and the proliferation of thymus derived (T) lymphocytes *in vitro* (McDevitt & Benacerraf 1969; Benacerraf & Germain 1978).

Class III genes code for serum proteins Ss (serum serological) and Slp (Sex-limited protein), and these molecules provide the C4 component of the classical complement pathway.

Minor histocompatibility antigens (non H-2) are controlled by genes scattered throughout the mouse genome. The intensity of skin graft rejection response to individual minor histocompatibility antigens is generally less than that seen when there are differences at the major histocompatibility complex, but cumulative non H-2 immunogenicity may on occasions equal that of the H-2 complex (Graff & Bailey 1973; Graff 1978). However, under normal physiological conditions, the functions of minor H antigens are thought to be non-immunological, and thus unrelated to the function of MHC antigens, which are primarily involved in "non-self" recognition processes (Lafferty & Woolnough 1977; Klein 1978).

CHAPTER TWO

MATERIALS & METHODS

CHAPTER TWO

EXPERIMENTAL ANIMALS

Inbred strains of mice were used in all experiments. For transplantation experiments, A.TL, B10.G, BALB/c, C57Bl/6, CBA/H and DBA/2 mice were obtained from the Animal Breeding Establishment at the John Curtin School of Medical Research. For the histocompatibility antigen labelling experiments (Chapter 6), A.TH, A.TL, B10.D2 and BALB/c animals were obtained from colonies maintained at the Austin Hospital (Victoria, Australia). C3H/HeJ mice were obtained from the Institute of Medical and Veterinary Sciences (Adelaide, Australia), while C57Bl/10Sn and B10.Br/SgSn mice were from the colonies maintained at Adelaide University. Animals were 8 - 16 weeks of age when used as recipients in transplantation experiments, while donors of adult tissues were 6 - 8 weeks old at sacrifice. In studies where adult mouse tissues were used for transplantation purposes, the donor and recipient strains were of the same sex. Experimental animals were housed either singly or in groups of 4 or less in wire-topped cages. All mice were provided with water *ad libitum* and the standard diet was Mecon mouse cubes (Fidelity Feeds, Murrumburrah, N.S.W.).

PREPARATION OF BALANCED SALT SOLUTIONS AND CULTURE MEDIA

Hanks Balanced Salt Solution was prepared under sterile conditions, according to the method of Hanks & Wallace (1949). $NaHCO_3$ was deleted, and the solution was buffered with N-2 hydroxy-ethyl piperazine-N^1-2 ethane sulphonic acid (HEPES) with a corrected pH of 7.4, and at a final concentration of 20mM. RPMI 1640 culture medium was prepared by dissolving 10.4g of RPMI 1640 powder (Microbiological Associates, U.S.A.) in double deionised water, and then adding 60ml of 1.4% $NaHCO_3$ solution. The volume was then made up to 1 litre, using double-deionised water, and the solution sterilised by filtration. A final pH of 7.4 was established by adding 2ml of 1M HEPES buffer (pH 7.4) to each 100ml of medium.

PREPARATION OF AVERTIN ANAESTHETIC SOLUTION

A 1ml aliquot of 2 methyl-2 butanol solution (Fluka AG, Switzerland) was added to 1gm of 2,2,2,-tribrom-ethanol (Fluka AG, Switzerland) in a 50ml flask, and mixed until all the solid was dissolved. The mixture was then diluted to a final volume of 50mls with hot (50^{o}) water, and then shaken vigorously. The solution was aliquoted in 5ml bottles, and frozen for storage. A suitable dose for mice was 1.25mls/100gm body weight, given by intraperitoneal injection.

PREPARATION OF CYCLOPHOSPHAMIDE FOR INJECTION

Cyclophosphamide (Endoxan-Asta) powder was diluted to a final

concentration of 10mg/ml by dilution with sterile distilled water, and injected intraperitoneally at a calculated dosage of 300mg/kg body weight at 4 and 2 days prior to removal of the donor pancreas or thyroid.

PREPARATION OF MOUSE PANCREATIC ISLETS FOR ORGAN CULTURE

This method is a derivation of that first described by Lacy and Kostianovsky (1967) for the isolation of rat pancreatic islets. Groups of 8 - 12 donor animals were anaesthetised with ether, and then killed by cervical dislocation. Following this, they were briefly but fully immersed in a solution of 70% ethanol, and then laid out in a laminar flow hood. The abdominal cavities were then opened, and all further isolation steps were carried out under sterile conditions. The pancreata were removed, and transferred to an autoclaved and siliconised 20ml glass scintillation vial. The tissue was diced for 30 seconds with a pair of dissecting scissors, and then washed in HEPES-buffered Hanks solution (pH 7.4). This solution was supplemented with 0.05% bovine serum albumin, pencillin (100u/ml), streptomycin (100 microgram/ml) and neomycin (100 microgram/ml). The minced tissue was allowed to settle at 4^{o} for 4 minutes, and floating debris was removed with a Pasteur pipette. After three 30 second dicings and subsequent washings, the chopped pancreatic tissue was transferred to a siliconised 15ml glass centrifuge tube, and allowed to settle for an additional 4 minutes at 4^{o}. The volume of chopped tissue was noted, and the tissue was then

concentration of 10mg/ml by dilution with sterile distilled water, and injected intraperitoneally at a calculated dosage of 300mg/kg body weight at 4 and 2 days prior to removal of the donor pancreas or thyroid.

PREPARATION OF MOUSE PANCREATIC ISLETS FOR ORGAN CULTURE

This method is a derivation of that first described by Lacy and Kostianovsky (1967) for the isolation of rat pancreatic islets. Groups of 8 - 12 donor animals were anaesthetised with ether, and then killed by cervical dislocation. Following this, they were briefly but fully immersed in a solution of 70% ethanol, and then laid out in a laminar flow hood. The abdominal cavities were then opened, and all further isolation steps were carried out under sterile conditions. The pancreata were removed, and transferred to an autoclaved and siliconised 20ml glass scintillation vial. The tissue was diced for 30 seconds with a pair of dissecting scissors, and then washed in HEPES-buffered Hanks solution (pH 7.4). This solution was supplemented with 0.05% bovine serum albumin, pencillin (100u/ml), streptomycin (100 microgram/ml) and neomycin (100 microgram/ml). The minced tissue was allowed to settle at 4^{o} for 4 minutes, and floating debris was removed with a Pasteur pipette. After three 30 second dicings and subsequent washings, the chopped pancreatic tissue was transferred to a siliconised 15ml glass centrifuge tube, and allowed to settle for an additional 4 minutes at 4^{o}. The volume of chopped tissue was noted, and the tissue was then

re-transferred to the glass scintillation vial. Collagenase (Boehringer Mannheim - Catalogue No. 103586) was freshly prepared in phosphate-buffered saline (PBS) at a concentration of 10mg/ml, and then filtered through a 22 micron Millipore filter. One ml of this solution was added to each 1ml volume of chopped pancreatic tissue, and the volume was made up to 5ml with HEPES-buffered Hanks solution. The pancreatic tissue was then digested at 37^{o} for 17 minutes, with the scintillation vial being fixed to an oscillating platform (200 cycles/minute) fitted within a heated water bath (Anax-Labmaster, Melbourne). The digest was then vigorously shaken by hand for an additional 15 - 20 seconds, in order to disperse any residual clumps of tissue. The enzymic digestion was slowed down by the addition of 15ml of chilled HEPES-buffered Hanks solution, and the digest allowed to settle for 4 minutes at 4^{o}. The supernatant liquid was removed, and the washing procedure repeated 2 more times. Individual isolated islets were located using a stereo microscope (Olympus SZ-111) set at 15 x magnification, and then removed from the digest with a fine siliconised pipette, and placed on ice in a petri dish containing HEPES-buffered Hanks solution. The islet yield averaged 40 islets per mouse pancreas. At the end of the isolation procedure, the islets were transferred by pipette to a petri dish held on ice and containing RPMI 1640 culture medium (Microbiological Associates, U.S.A.) supplemented with 10% heat-inactivated foetal calf serum (Flow Laboratories, Australia) and antibiotics.

ISOLATION OF FOETAL PANCREAS

Foetal pancreata were obtained from 16 - 17 day old BALB/c foetuses conceived following an overnight mating. The day following such a mating was designated day 0 for the purpose of determining gestational age. Pregnant females were killed by cervical dislocation, and the foetuses were delivered under sterile conditions by hysterotomy. Foetuses were killed by decapitation and their abdomens opened with a scalpel. In each case the pancreas was identified and removed, and placed into a sterile petri dish containing HEPES-buffered Hanks solution. This was placed on ice prior to commencement of culture.

ORGAN CULTURE OF MOUSE PANCREATIC ISLETS

Groups of approximately 50 isolated islets were transferred to 35mm Falcon type 1008 hydrophobic petri dishes (Andersson, 1978) containing 2ml of culture medium (RPMI 1640 plus 10% heat-inactivated foetal calf serum). These were placed within a humidified gas-tight portable incubator (Figure 2.1) which was sealed and then charged for 10 minutes with a 95% O_2 and 5% CO_2 gas mixture flowing at 1 litre a minute. The incubator was then placed on an orbital platform (Gallenkamp SGM-300) that rotated at 50 - 60 cycles a minute. Islets would usually aggregate within an hour or two, but were left on the platform overnight, so as to consolidate their adhesion. Ambient air temperature was maintained at 37^{o}. Medium was

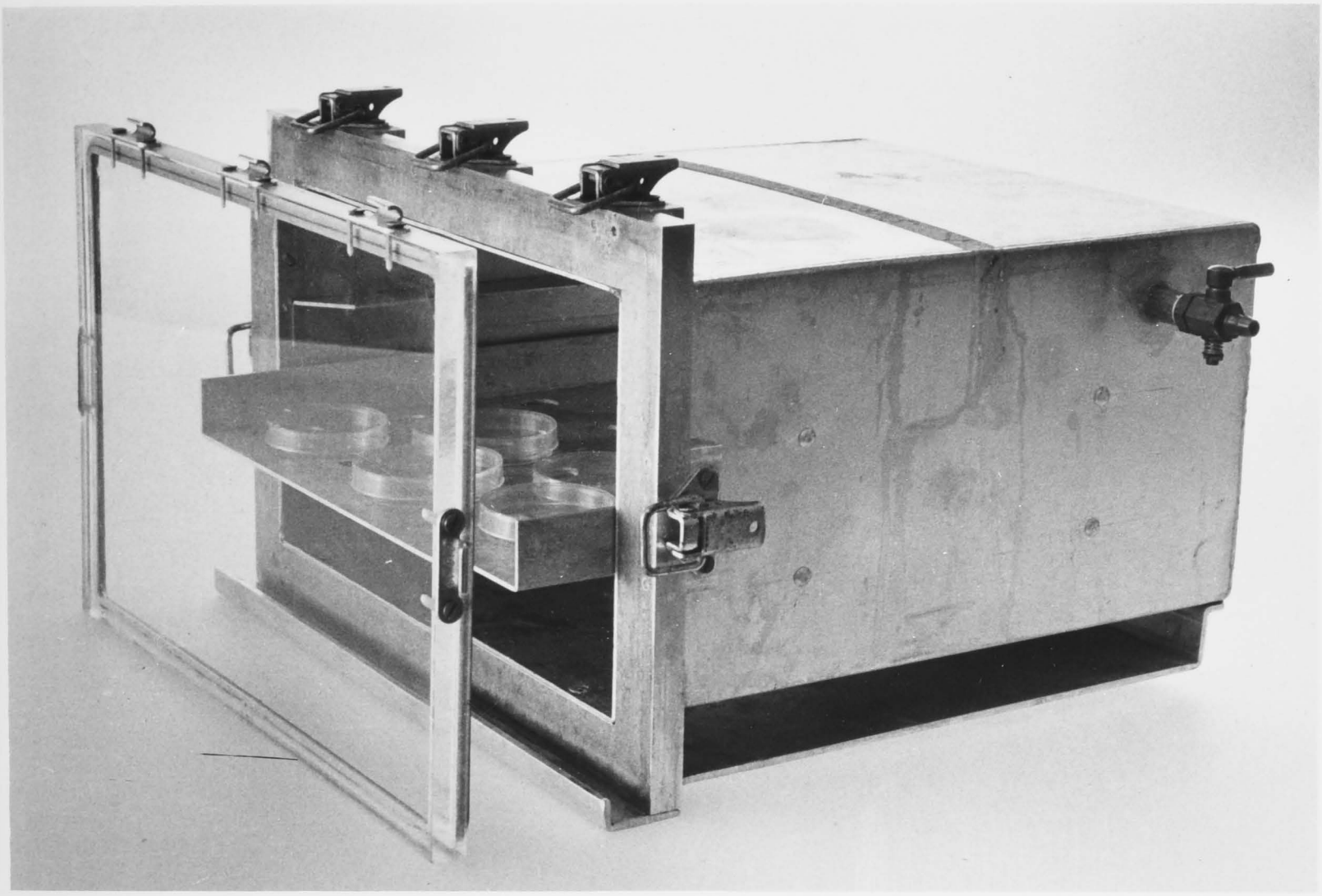


Figure 2.1 A gas tight metal incubation box designed for culture of tissue in a 95% oxygen gas phase. Note the perspex door, which enables observation of islet aggregation.

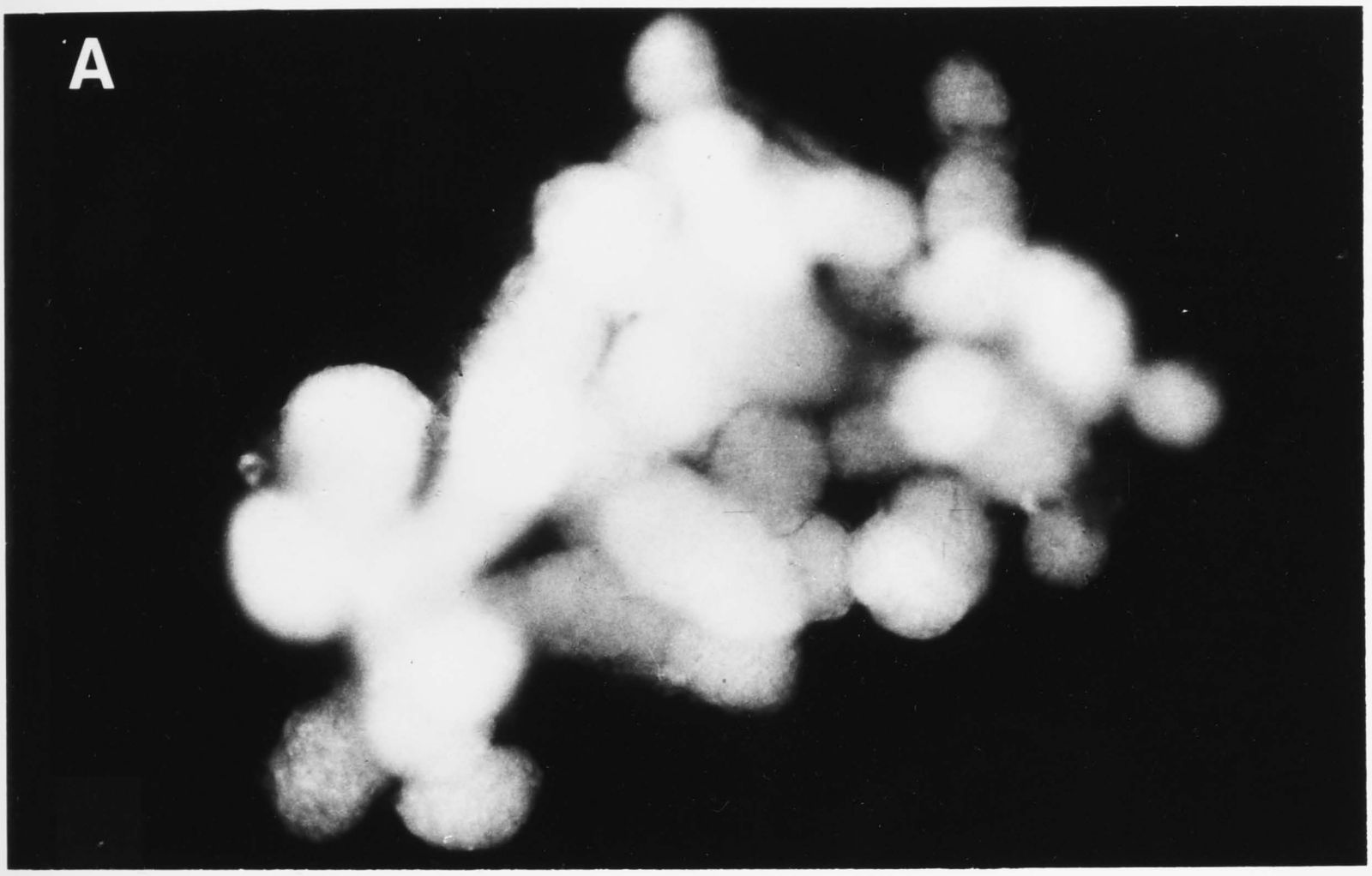
changed 3 times a week; on the first and subsequent changes, medium sufficient to just cover the bottom of the dish (0.75ml) was added. The islet aggregates (Figure 2.2A) coalesced over a 4 - 7 day culture period to form tight clusters (Figure 2.2B) that could be manipulated for transplantation with fine forceps or a siliconised Pasteur pipette.

PREPARATION OF UNCULTURED ISLET CLUSTERS

Loose clusters of uncultured islets were prepared by placing 50 adult BALB/c islets in 35mm hydrophobic culture dishes (Falcon 1008), each containing 2ml of RPMI 1640 culture medium supplemented with 10% heat-inactivated foetal calf serum, and antibiotics (Pencillin 100u/ml, streptomycin 100 microgram/ml, and neomycin 100 microgram/ml). The islets were maintained in a gas phase of 5% CO_2 in air at 37^{o} and rotated on an orbital platform (Gallenkamp SGM-300) at 50 - 60 cycles a minute for 4 hours, by which time loose clusters of islets had formed. Medium was subsequently changed 3 times a week, with sufficient media to just cover the bottom of the dish (0.75ml) being added.

ORGAN CULTURE OF FOETAL PANCREAS

Individual foetal pancreata were transferred to 35mm hydrophobic petri dishes (Falcon 1008) containing 1ml of culture medium, and then cultured in a gas-tight incubator (Figure 2.1) for 10 days at 37^{o} in a humidified gas phase of 95% O_2 and



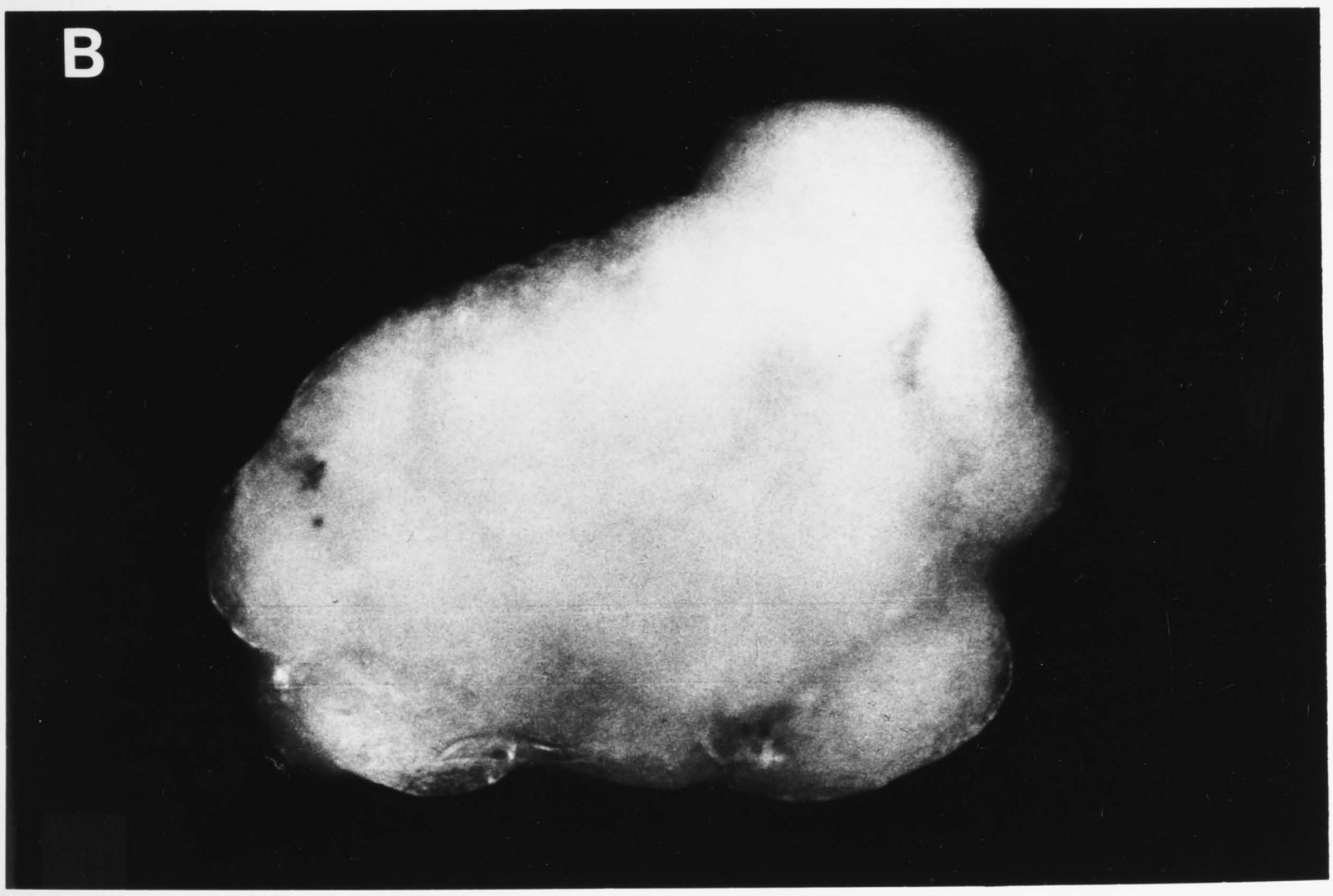


Figure 2.2A An aggregate of approximately 50 BALB/c islets at the commencement of cluster formation (x 100).

Figure 2.2B An islet cluster after 7 days in culture. Note the coalescence of individual islets (x 100).

5% CO_2. Pancreata destined for a 17 day culture period were initially cut into 3 segments, these being cultured in a single petri dish. Culture medium was changed three times a week in all cases.

ORGAN CULTURE OF MOUSE THYROID LOBES

Thyroid lobes taken from normal donors, or from donors pretreated with cyclophosphamide (300mg/kgm) on days 4 and 2 prior to gland removal were cultured at 37^{o} in Falcon 1008 bacteriological petri dishes containing 2mls of RPMI 1640 culture medium supplemented with 10% v/v heat in-activated foetal calf serum (HIFCS) and antibiotics. The gas phase used was 95% O_2 and 5% CO_2, and the medium was changed 3 times a week. The duration of organ culture was 7 or 14 days.

TRANSPLANTATION OF CULTURED PANCREATIC ISLET CLUSTERS AND FOETAL PANCREAS

Graft recipients were anaesthetised with intraperitoneal injection of Avertin, and their left flanks shaved with a power shaver. The shaved area was then swabbed with 70% ethanol, and a 2cm skin incision made over the site of the left kidney with a scalpel. Small dissecting scissors were used to open the abdominal wall, and full exposure of the kidney was achieved by lifting it clear of the abdominal cavity with curved forceps. A small nick in the renal capsule was then made with a 20 gauge needle, and the

capsule gently separated from the underlying renal parenchyma with fine straight jeweller's forceps. A subcapsular tunnel was fashioned, and the graft was then passed down the tunnel to its blind end, using either forceps or a siliconised Pasteur pipette. The capsule tended to close over the graft, and thereby prevented loss of the tissue into the peritoneal cavity. The wound was closed with Autoclips, and the animal revived by gentle warming under a 60 watt incandescent lamp.

EVALUATION OF TRANSPLANTED TISSUE

Tissue transplants were evaluated by histologic examination. At varying times after transplantation, recipient mice were killed by cervical dislocation. The kidney carrying the transplanted islet tissue was fixed in 10% formal-saline, and paraffin sections were stained with hematoxylin and eosin or with aldehyde-fuchsin. The latter selectively stains the insulin-containing granules of the islet beta cells. Tissue sections were examined to determine the degree of islet damage and mononuclear cell infiltration in and around the transplanted tissue.

RESECTION OF THYROID LOBES

Donor animals were killed with ether, the necks swabbed with 70% alcholol, and a 2cm mid-line incision made in the neck with a number 10 scalpel blade. The wound was held

open with a small retractor, and the submandibular glands and anterior cervical muscle groups separated using blunt dissection. The two lobes of the thyroid gland were then easily identified, one lobe lying on either side of the trachea. The lobes were removed using blunt dissection with straight edged scissors, and placed into RPMI culture medium buffered to pH 7.4 prior to their dissociation in collagenase.

ESTIMATION OF BLOOD GLUCOSE LEVELS

The Boehringer-Mannheim GOD-Perid test kit for glucose estimations was used. Whole blood (10 microlitres) was collected from the tip of the tail, and placed into a small Beckman centrifuge tube containing 100 microlitres of 0.66M perchloric acid. Samples were briefly vortexed, and the deproteinised blood centrifuged for 90 seconds in a Beckman Minifuge. Two 20 microlitre aliquots were taken from each supernatant, and placed into blood collection tubes. GOD enzyme reagent (1ml) was added to each tube. These were vortexed and then placed in a 37^{o} water bath for 15 minutes. Glucose content was determined against a glucose standard using a spectrophotometer set at 436 nanometres. Results were expressed as mmol glucose/litre whole blood. The lower limit for this assay was 0.2mmol/1.

ESTIMATION OF URINARY GLUCOSE LEVELS

The Boehringer-Mannheim GOD Perid test kit was used. Urine

was collected over a 24 hour period by placing individual mice in a small cage with a wire-mesh bottom. Voided urine passed into a collection cylinder. At the end of the collection period the cage was carefully washed down, the washings collected, and made up to a final volume of 1 litre. Aliquots (10 microlitres) were pipetted into small Beckman centrifuge tubes containing 100 microlitres of 0.66M perchloric acid, and then centrifuged for 90 seconds. Two 20 microlitre aliquots were taken from each supernatant, and placed in blood collection tubes. Glucose content was then assayed using the method described for blood sugar estimations. Results were expressed as mmol glucose/24 hours urine. The lower limit of this assay was 0.2mmol glucose/24 hour sample. Normal CBA mice failed to excrete detectable amounts of glucose.

PREPARATION OF PERITONEAL CELLS

Donor mice were killed by cervical dislocation, and their abdomens swabbed with 70% alcohol. An incision was made in the skin of the anterior abdominal wall, and the skin gently stripped back to expose the peritoneum. The peritoneal cavity was then irrigated with 2.5ml of HEPES-buffered Hanks solution supplemented with heparin (20u/ml) and the resulting cell suspension centrifuged at 800g for 5 minutes. The cell pellet was then resuspended in 1ml of heparin-free HEPES-buffered Hanks solution. Viable cells were counted using a trypan blue dye exclusion test. Equal volumes (0.1ml)

of cells and 0.5% trypan blue dye in phosphate-buffered saline were mixed, and an aliquot counted in a haemocytometer.

DISSOCIATION OF THYROID GLANDS AND ISLETS OF LANGERHANS

Mouse thyroid glands were collected in groups of 10 (20 lobes) in cold culture medium, drained, minced finely with scissors, and suspended in 10ml of a solution of 1mg of collagenase per ml (Sigma Chemical Co.; Type III) in cold culture medium. The thyroid mince was incubated in a screw-capped tube for 20 min at 37^{o} with frequent shaking. The digest was collected by brief centrifugation, washed once in cold culture medium and fixed by suspension in 4ml of periodate-lysine-paraformaldehyde (PLP) fixative for 3 hr at 4^{o} (McLean & Nakane 1974). The PLP fixative has no detectable effect on either H-2 complex or la^{k} antigen labelling (Kirby & Parr 1979; Parr & McKenzie 1979) and was prepared as described previously (Parr 1979a). The fixed thyroid digest, consisting at this stage largely of isolated follicles and short segments of vessels, was washed for 10 min in phosphate-buffered saline (PBS) and 20 min in PBS containing 0.02% ethylene diamine tetra-acetic acid (EDTA). The washed digest was suspended in 2ml of 0.25% trypsin (Sigma Chemical Co.; Type II) in PBS-EDTA for 15 min at 37^{o}, mixed with an equal volume of cold 5% bovine serum albumin in PBS and then pipetted vigorously for a few seconds to dissociate the cells. The cells were collected by centrifugation and resuspended in 10% non-

immune rabbit serum in PBS for 30 minutes at 4^{o} before labelling. Cell suspensions from mouse islets of Langerhans were prepared as described previously (Parr 1979a).

PREPARATION OF ANTIBODY-FERRITIN CONJUGATE

To prepare the antibody-ferritin conjugate, the IgG fraction of a rabbit anti-mouse gamma-globulin serum was isolated on a protein A column and conjugated to ferritin with glutaraldehyde in a two-step procedure as described previously (Parr 1979a).

PREPARATION OF ANTISERUM

Allo-antiserum specific for the whole H-2^{b} complex was produced in (B10.BR/SgSn x C3H/HeJ) F_1 hybrid females by immunising with C57B1/10Sn spleen cells. A total of 6 weekly immunisations were given, beginning with 0.05 spleen/mouse, and ending with 0.3 spleen/mouse. Antiserum titres of 1/60 were demonstrated by haemolysis assay (Parr & Oei 1973) with rabbit complement (Koene & McKenzie 1973). Antiserum specific for the Ia^{k} region of the H-2 complex was produced by immunising A.TH mice with lymphoid cells from A.TL mice. After absorption with lymphoid cells of BALB/c and B10.D2 mice, this antiserum had a cytotoxic titre (rabbit complement dependent) of 1/1250 for B10.BR/SgSn, and less than 1/2 for BALB/c. The absorbed antiserum labelled Ia antigens on B10.BR/SgSn lymphocytes and jejunal absorptive cells, but

B10.D2 cells failed to label, and as such, provided a suitable congenic control for Ia^k labelling studies.

LABELLING PROCEDURE AND PREPARATION FOR ELECTRON MICROSCOPY

Cell pellets from thyroid glands and islets of Langerhans were approximately 1 microlitre in volume. They were suspended in 200 microlitres of a 1/10 dilution of mouse antiserum in 10% rabbit serum for 30 minutes at 4^{o}, washed twice in PBS containing 1% bovine serum albumin, incubated for 30 minutes in 200 microlitres of conjugate, washed twice in PBS containing 1% bovine serum albumin, twice in plain PBS, and fixed in 2.5% glutaraldehyde (EM grade; Taab Laboratories, Reading, England) in 0.10M cacodylate buffer (pH 7.4) for 2 hours at 20^{o}. The cells were fixed as a pellet by layering the fixative gently over the cells at the bottom of a centrifuge tube. After about 10 minutes the cell pellet could be raised into the fixative. Cell pellets were washed for 17 hours in cacodylate buffer and 20 minutes in distilled water, postfixed for 1 hour at 4^{o} in osmium-ferrocyanide, washed, dehydrated in ethanol, and embedded in Epon-araldite. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope.

PREPARATION OF FREUNDS COMPLETE ADJUVANT FOR INJECTION

Freunds complete adjuvant (1ml) was emulsified in an equal volume of PBS by rapid passage of the mixture back and forth

between two 5ml plastic syringes connected via a 2-way stop-cock. Lack of dispersal of a drop of the mixture indicated that satisfactory emulsification had occurred. A volume of 0.2ml of the emulsion was injected intra-peritoneally into the animals under test.

DETERMINATION OF THE NORMAL RANGE FOR NON-FASTING BLOOD GLUCOSE LEVELS

Normal non-fasting male BALB/c mice (20 animals) and CBA mice (30 animals) were bled from the tail vein between 9a.m. and 10a.m., and the blood glucose levels measured. The 95% confidence intervals for non-fasting blood glucose levels were then calculated (Swinscow 1980).

PREPARATION OF ACETATE BUFFER

A stock solution of acetate buffer (pH 4.2) was produced by adding 30.5mls of 0.2M acetic acid to 19.5mls of 0.2M sodium acetate, and 0.9g of NaCl was dissolved in this solution. The final volume of 100ml was reached by adding distilled water.

INDUCTION OF STREPTOZOTOCIN DIABETES

Immediately prior to injection, 60mg of streptozotocin (Boehringer Mannheim, Germany) was dissolved in 2ml of acetate buffer. The solution was drawn up in a 1ml syringe, and injected into the tail vein of recipient CBA animals via a 26g needle, at a dosage of 300mg/kg body weight (Weber *et al* 1976). Animals received islet grafts 14 days after induction of diabetes.

CHAPTER THREE

TRANSPLANTATION OF MOUSE PANCREATIC ISLETS TO NON-IMMUNOSUPPRESSED ALLOGENEIC RECIPIENTS

CHAPTER THREE

INTRODUCTION

There is now evidence that transplantation antigens on the surface of tissue parenchymal cells are not the major barrier to allotransplantation (Lafferty & Woolnough 1977), and that haematogenous cells carried within the transplanted tissue play a central role in the induction of allograft reactivity. Cells that express the Stimulator (S+) phenotype are required for the induction of specific allograft immunity (Talmage *et al* 1977), and the activation of blood cells within the transplant itself can give rise to local inflammatory responses that cause damage to the transplanted tissue (Lafferty & Woolnough 1977).

It has been shown with mouse thyroid and parathyroid that maintenance of these tissues in organ culture in a gas phase of 95% O_2 and 5% CO_2 for a period of 21 - 28 days before transplantation results in their long-term acceptance by normal allogeneic recipients (Lafferty & Woolnough 1977). The period of organ culture can be considerably shortened if transplant donors are treated with cyclophosphamide before the removal of the tissues for transplantation

(Lafferty & Woolnough 1977). The cultured tissue is antigenic, and can be rejected if the immune system of the recipient animal is activated with either leucocytes of donor origin (Talmage *et al* 1976), or an uncultured thyroid of donor origin (Lafferty & Woolnough 1977). These findings have been confirmed by Sollinger *et al* (1977). Thus, this treatment of tissues before transplantation reduces their immunogenicity for an allogeneic host but does not eliminate their expression of transplantation antigen.

Since endocrine pancreas transplantation has potential clinical relevance in the treatment of Type I diabetes mellitus, and most experimental data show that pancreatic islets are highly immunogenic (see reviews by Karl & Lacy 1977; Jonasson & Hoversten 1978; Schulak & Reckard 1978; Barker, Naji & Silvers 1980), the studies that follow were carried out to determine whether mouse pancreatic islets could be conditioned by organ culture prior to allotransplantation across major and minor histocompatibility barriers.

RESULTS

Survival of Uncultured Islets Under the Kidney Capsule of Isogeneic Recipient Mice

Approximately 50 individual uncultured islets from normal or cyclophosphamide pretreated BALB/c donors were transplanted under the left kidney capsule of BALB/c recipients,

and examined histologically at varying times after transplantation. Islets from both normal and cyclophosphamide pretreated donors behaved in the same way when grafted to isogeneic recipients, with revascularisation of the islets being evident at about 4 days following transplantation. When stained with aldehyde-fuchsin, positively stained beta cells were seen in the isografted tissue (Figure 3.1).

The Allograft Response to Uncultured Pancreatic Islets

In the first series of experiments, approximately 50 isolated BALB/c ($H\text{-}2^d$) pancreatic islets from normal or cyclophosphamide pretreated donors were grafted under the renal capsules of normal CBA ($H\text{-}2^k$) recipients. Groups of animals were killed 4, 7 and 14 days after transplantation, and their grafts examined histologically. Quantitation of graft rejection was on the basis of the degree of mononuclear cell infiltration into the graft, and the extent of disruption of graft integrity (Table 3.1).

There was a violent allograft response to normal islets, with 4 day old allografts showing light to heavy infiltration with mononuclear cells. Numerous small lymphocytes, lymphoblasts, and macrophages were present, as was the occasional polymorph. At this stage the islets were generally intact, although some contained small pockets of lymphocytes. By day 7, the centre of the islets had

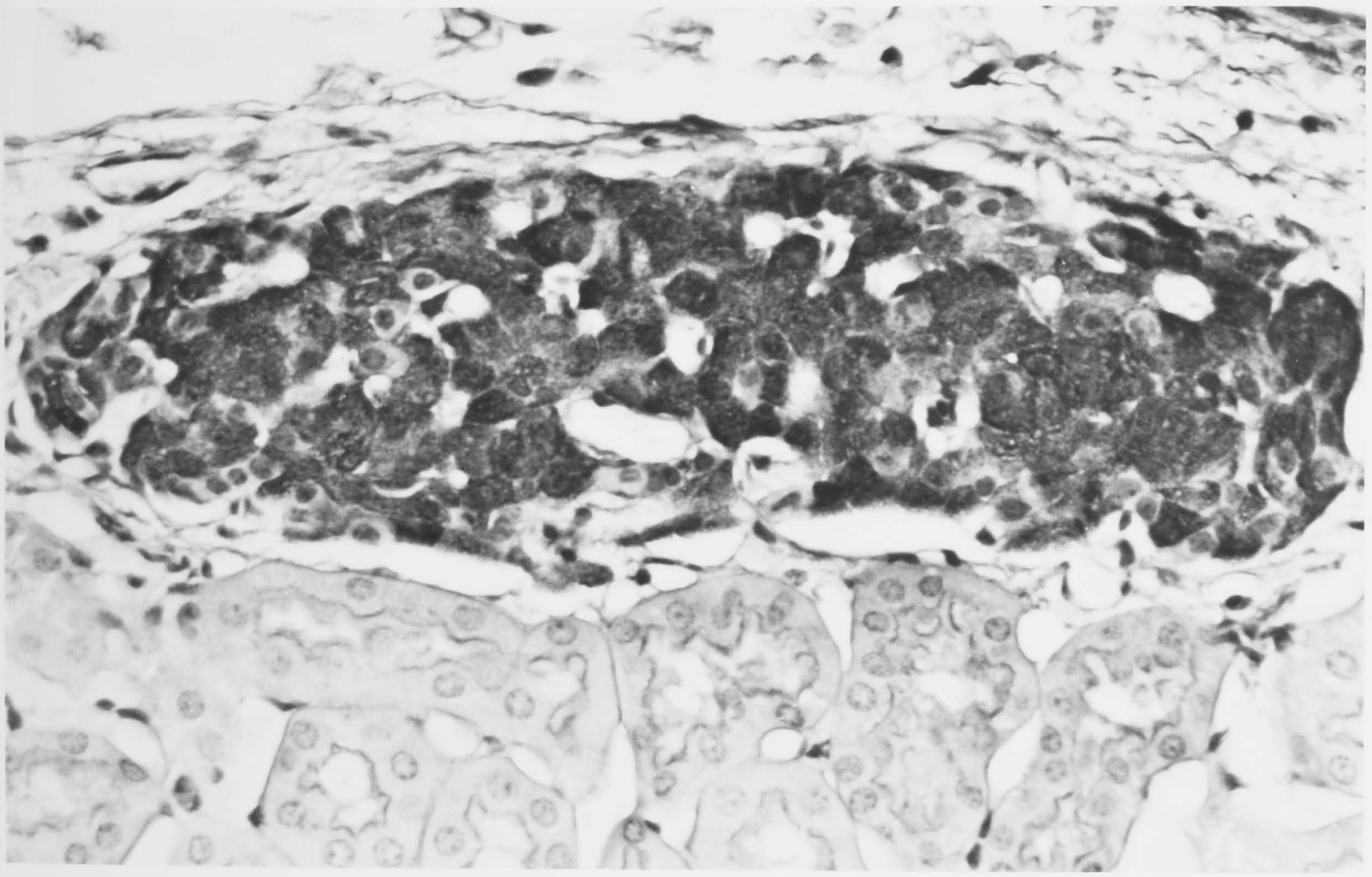


Figure 3.1 BALB/c islets transplanted to BALB/c recipients and examined 21 days following transplantation (aldehyde-fuchsin x 1370)

TABLE 3.1 Protocol for histological scoring

(a) Mononuclear cell infiltration of islets and surrounding tissue

0	no infiltration
+	light infiltration around transplanted tissue
++	moderate infiltration around transplant, and a few mononuclear cells within islets
+++	heavy infiltration around graft, and pockets of lymphocytes within islets
++++	heavy infiltration around transplant, and islets penetrated by mononuclear cells with severe disruption of islet architecture

(b) Integrity of transplanted islets

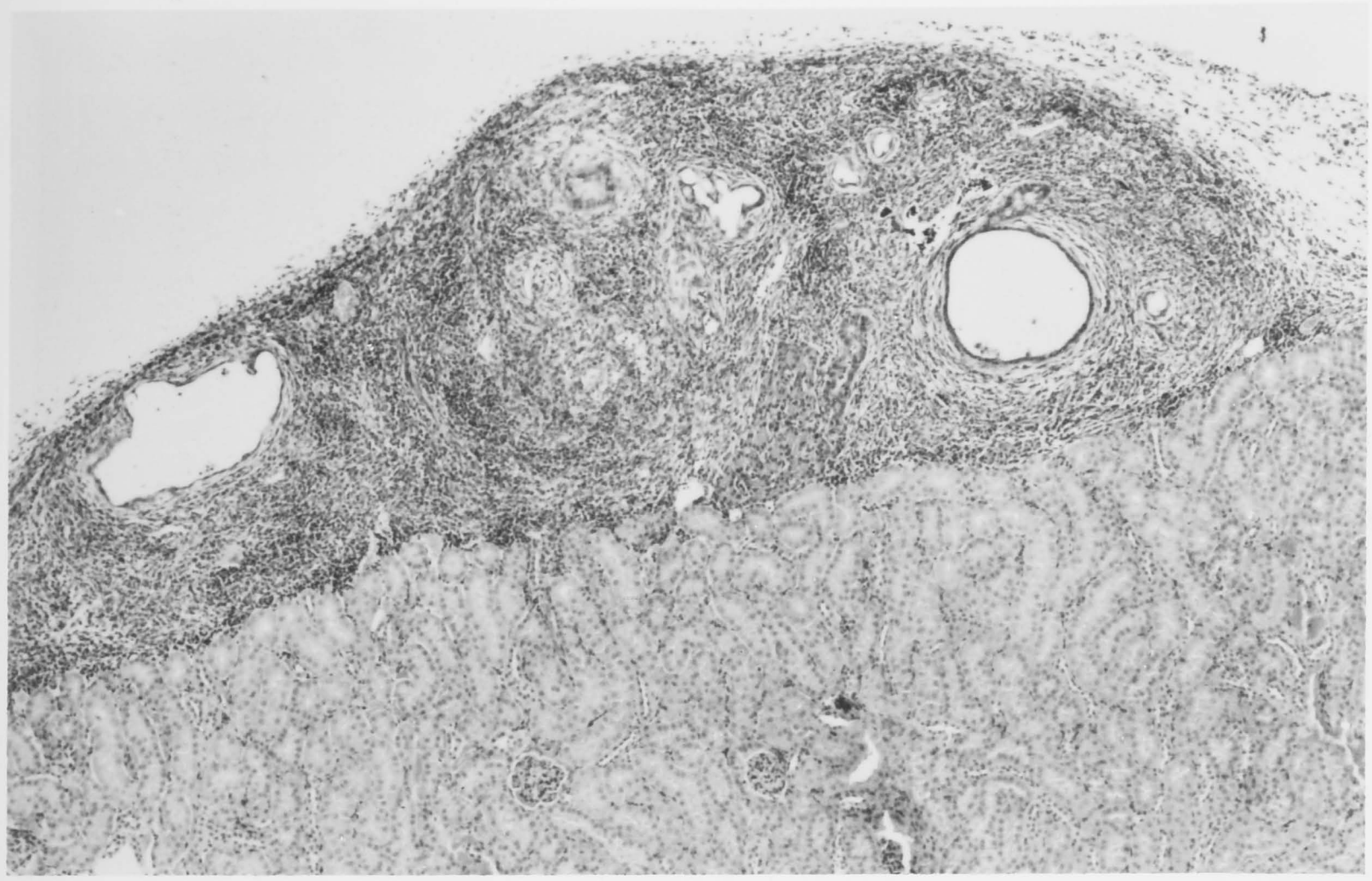
+++++	islets completely intact
++++	outer surfaces disrupted
+++	outer surfaces disrupted and centres of islets disorganised
++	entire islets disorganised
+	islets reduced to remnants
0	no islet tissue seen

become disorganised by infiltrating lymphoid cells, and graft rejection was complete by day 14, when only scattered fragments of islet tissue were seen at the graft site (Figures 3.2 and 3.3).

Donor pretreatment with cyclophosphamide reduced the intensity of the allograft response. By day 14 there was an obvious response to the grafted tissue, but islets from pretreated donors generally underwent a more chronic rejection process than islets from normal donors (Table 3.2). One allograft examined 21 days after transplantation still contained intact islets which showed prominent staining of beta cells with aldehyde-fuchsin (Figure 3.4).

Allograft Response to In Vitro Cultured Islet Tissue (Donors Pretreated with Cyclophosphamide)

Preliminary experiments had shown that isolated islets rapidly disintegrated when cultured in a gas phase of 95% O_2 and 5% CO_2. This effect has been observed by others (Lacy, Davie & Finke 1980). Attempts were then made to enlarge the tissue mass by aggregating the islets, and the best results were achieved when the islets were initially aggregated on an orbital platform. It was found that these aggregates survived well when cultured in a 95% O_2/5% CO_2 gas phase, and that they coalesced to form amorphous masses of tissue within 3 - 4 days of the commencement of culture. These islet clusters generally survived in organ culture for up to 21 days, with histological examination of the tissue showing the presence of numerous



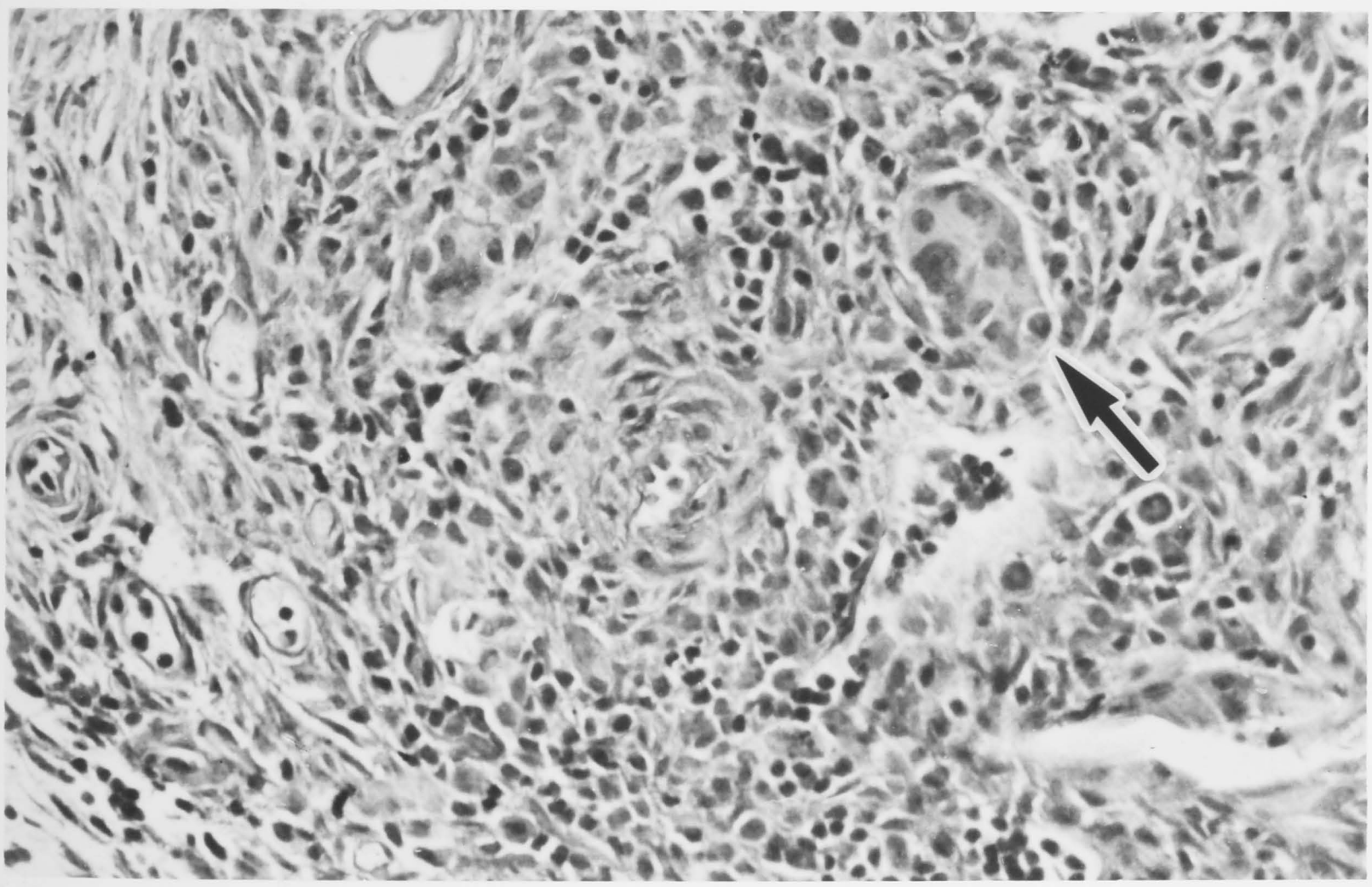


Figure 3.2(Top) BALB/c islets transplanted to a CBA recipient and examined 14 days after transplantation. There is a marked allograft response. (haematoxylin and eosin x 550)

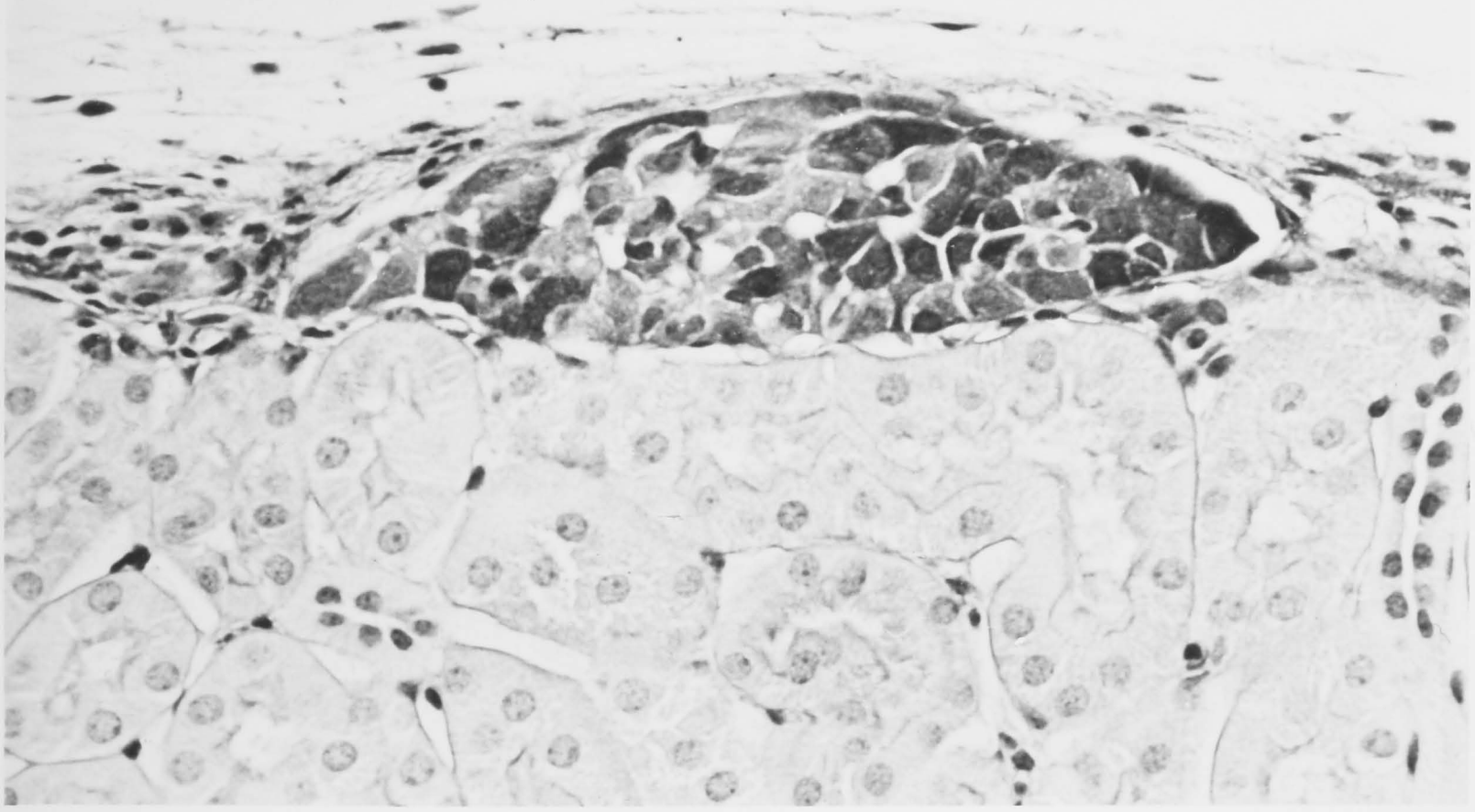
Figure 3.3(Bottom) BALB/c islets transplanted to a CBA recipient and examined 14 days after transplantation. Note remnants of islet tissue (arrows). (aldehyde-fuchsin x 1370)

TABLE 3.2 Histological appearance of islets from normal or cyclophosphamide-treated BALB/c donors after transplantation to the kidney capsule of CBA recipient mice

Donor pretreatment	Time after transplantation (days)	Histological Score	
		Mononuclear cell infiltration	Integrity of islets
None	4	+	++++
		+++	+++
		++	++++
		++	+++++
Cyclophosphamide	4	0	+++++
		0	+++++
		+	+++++
		0	+++++
None	7	++	+++
		+++	+++
		++++	+
Cyclophosphamide	7	++	++++
		+	++++
		++	+++++
None	14	++++	+
		++++	+
		++++	+
		++++	+
Cyclophosphamide	14	+	++++
		++++	+
		+	++
		++	++
		++	++++
		++	+++

aldehyde-fuchsin stained beta cells (Figure 3.5). In contrast, islet clusters cultured in air and 5% CO_2 contained large areas of necrosis after 7 days in culture, making them less suitable for transplantation (Figure 3.6). When cultured BALB/c islets from cyclophosphamide-treated donors were transplanted under the kidney capsule of normal CBA mice, little or no allograft response was observed (Table 3.3). A culture period of 4 days was sufficient to condition the islets for transplantation, but a culture period of 7 days was preferred as the islets had by this time formed tight clusters that were much easier to manipulate than the somewhat loose aggregates produced after 4 days in culture.

Fourteen days after transplantation, all 15 cultured allografts examined were intact, and had obvious aldehyde-fuchsin staining of cells. A further 14 allografts were removed 28 days after transplantation. The grafts were very well vascularised (Figure 3.7) and on histological examination only one had any sign of islet damage. A further 5 CBA mice received 7 - 12 day cultured islets from cyclophosphamide treated BALB/c donors, and the grafts examined after 84 days. All grafts were well vascularised, and histological examination showed that none of the grafts had induced an allograft response. Aldehyde-fuchsin staining demonstrated the presence of numerous beta cells in all the grafts (Figure 3.8).



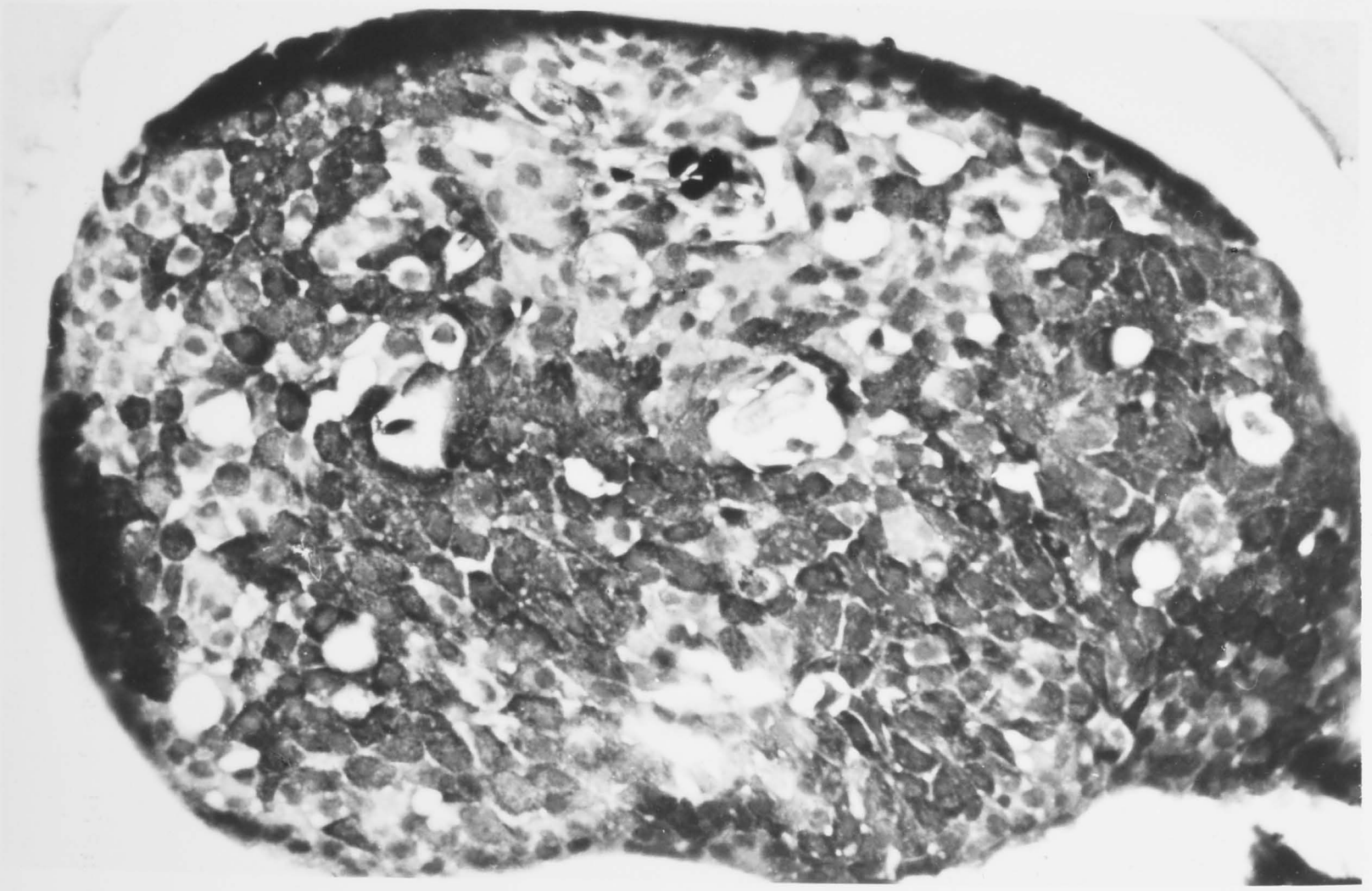


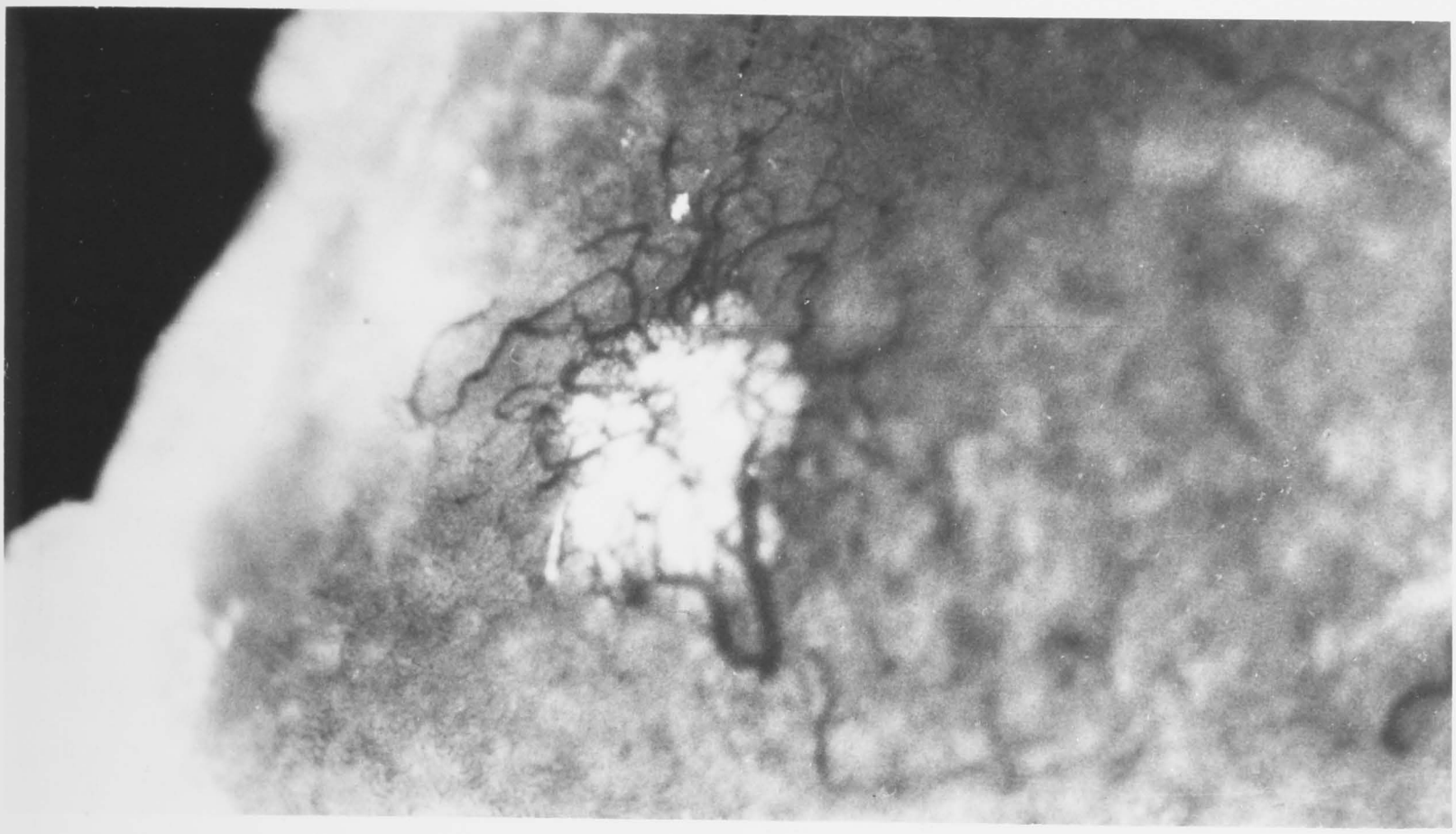
Figure 3.4(Top) BALB/c islets from cyclophosphamide pre-treated donors transplanted to a CBA recipient and examined 21 days after transplantation. (aldehyde-fuchsin x 1370)

Figure 3.5(Bottom) Section of an islet cluster after 7 days in organ culture (90% O_2 and 5% CO_2) showing presence of darkly stained beta cells. (aldehyde-fuchsin x 1370)

Figure 3.6 Sections of islet clusters after 7 days organ culture (air and 5% CO_2). Note central necrosis in all specimens. (haematoxylin and eosin x 400)

TABLE 3.3 Histological appearance of cultured islets after transplantation under the kidney capsule of CBA recipient mice. BALB/c donor mice were pre-treated with cyclophosphamide (300mg/kg body weight) 4 and 2 days prior to isolation of islets

Duration of Culture (Days)	Time after transplantation (Days)	Histological Score	
		Mononuclear cell infiltration	Integrity of islets
4	14	0	+++++
		0	+++++
		+	+++++
		+	+++++
		+	+++++
4	28	0	+++++
		0	+++++
		0	+++++
		0	+++++
7	14	0	+++++
		+	+++++
		+	+++++
		0	+++++
		+	+++++
		0	+++++
		+	+++++
7	28	0	+++++
		+	+++++
		+	+++++
		0	+++++
		0	+++++
12	14	0	+++++
		0	+++++
		0	+++++
		0	+++++
		0	+++++
		+	+++++
12	28	0	+++++
		0	+++++
		++	++++
		0	+++++
		0	+++++



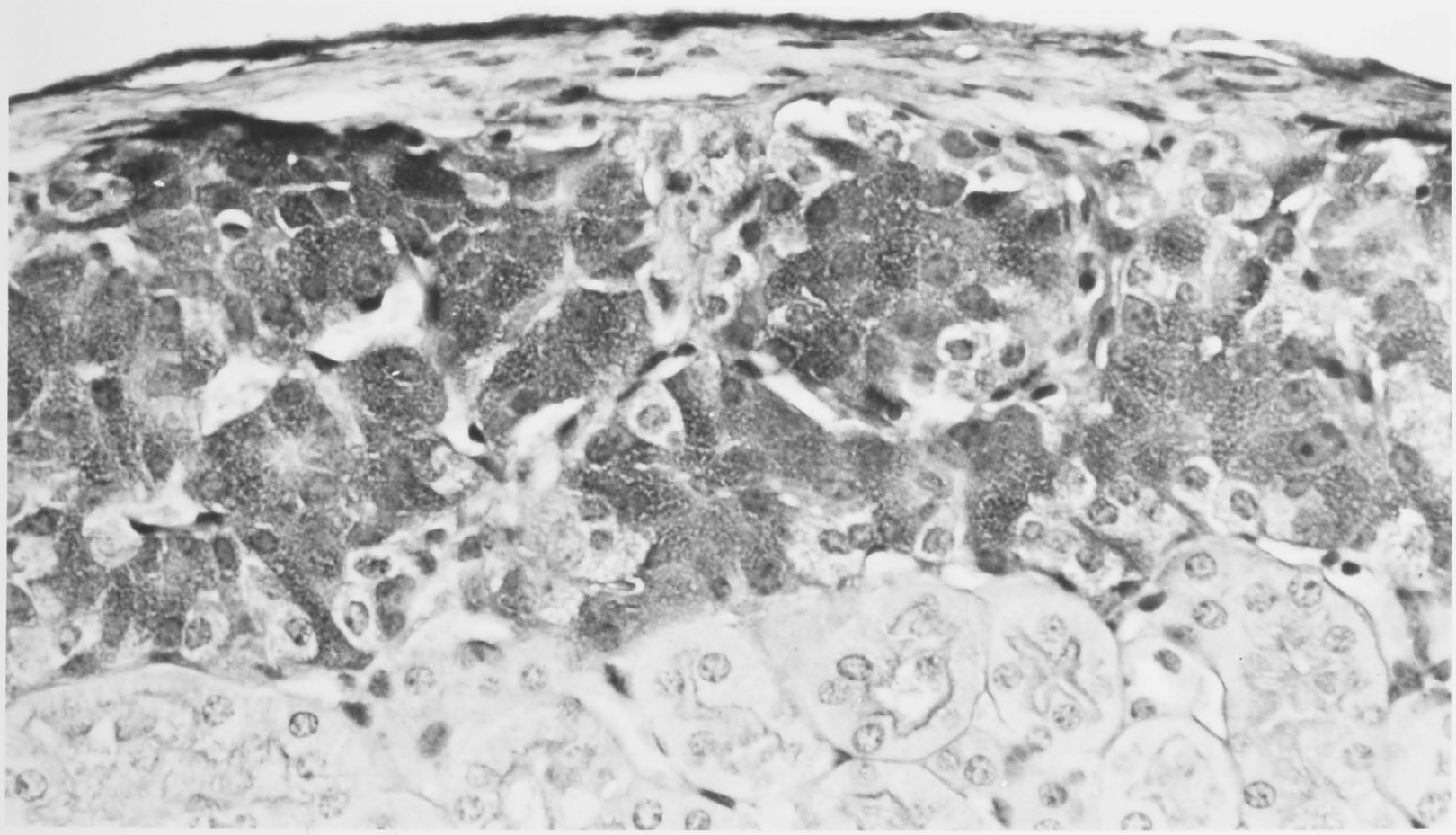
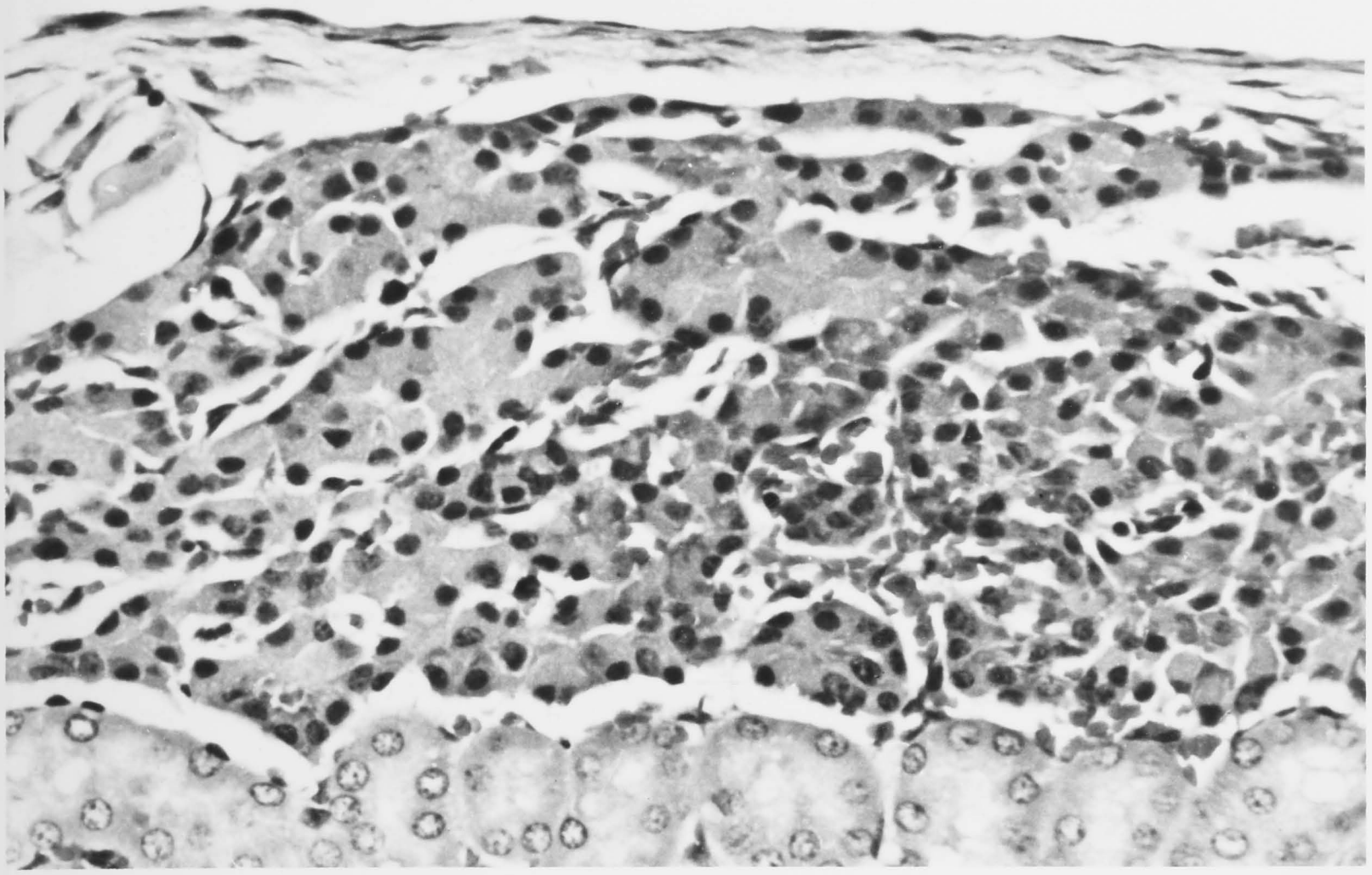


Figure 3.7(Top) A cluster of approximately 50 BALB/c islets 28 days after transplantation under the kidney capsule of a CBA recipient. Note the extensive revascularisation of the graft. The islet tissue was conditioned for transplantation by cyclophosphamide treatment of the tissue donor, and a 7 day period of organ culture (x 50)

Figure 3.8(Bottom) An allograft of BALB/c islets sited under the renal capsule of a CBA host, and examined 84 days following transplantation. The tissue was conditioned by cyclophosphamide pre-treatment of the donor and cultured for 7 days prior to transplantation.(aldehyde-fuchsin x 1370)

Allograft Response to In Vitro Cultured Islet Tissue (Donors not Pretreated with Cyclophosphamide)

Islet clusters derived from normal BALB/c mice that were not treated with cyclophosphamide were then cultured for 7 days, and these allografts were examined 14, 28 and 420 days after grafting to normal CBA recipients (Table 3.4). Graft survival was 100% for all 3 groups, and all grafts stained well with aldehyde-fuchsin and haemotoxylin and eosin (Figures 3.9 and 3.10). In these experiments, day 0 islet allograft controls were aggregated on the orbital platform in a gas phase of air and 5% CO_2 at 37^{o} for 4-6 hours prior to grafting, as it was often difficult to place compact collections of freshly isolated islets under the kidney capsules of recipient animals. The transplantation of loose aggregates with a Pasteur pipette solved this problem. The aggregates evoked a somewhat less vigorous host response than that previously seen with non-aggregated uncultured islet allografts; nevertheless, all showed evidence of partial or complete rejection by 28 days after transplantation (Figure 3.11). It is clear from this data that the organ culture of pancreatic islets from either normal or cyclophosphamide pretreated BALB/c ($H\text{-}2^{d}$) donors in a 95% O_2 and 5% CO_2 gas phase nullifies their immunogenicity and leads to their prolonged survival in a normal CBA ($H\text{-}2^{k}$) host, and that cyclophosphamide pretreatment of the donor is not necessary when this strain combination is used.



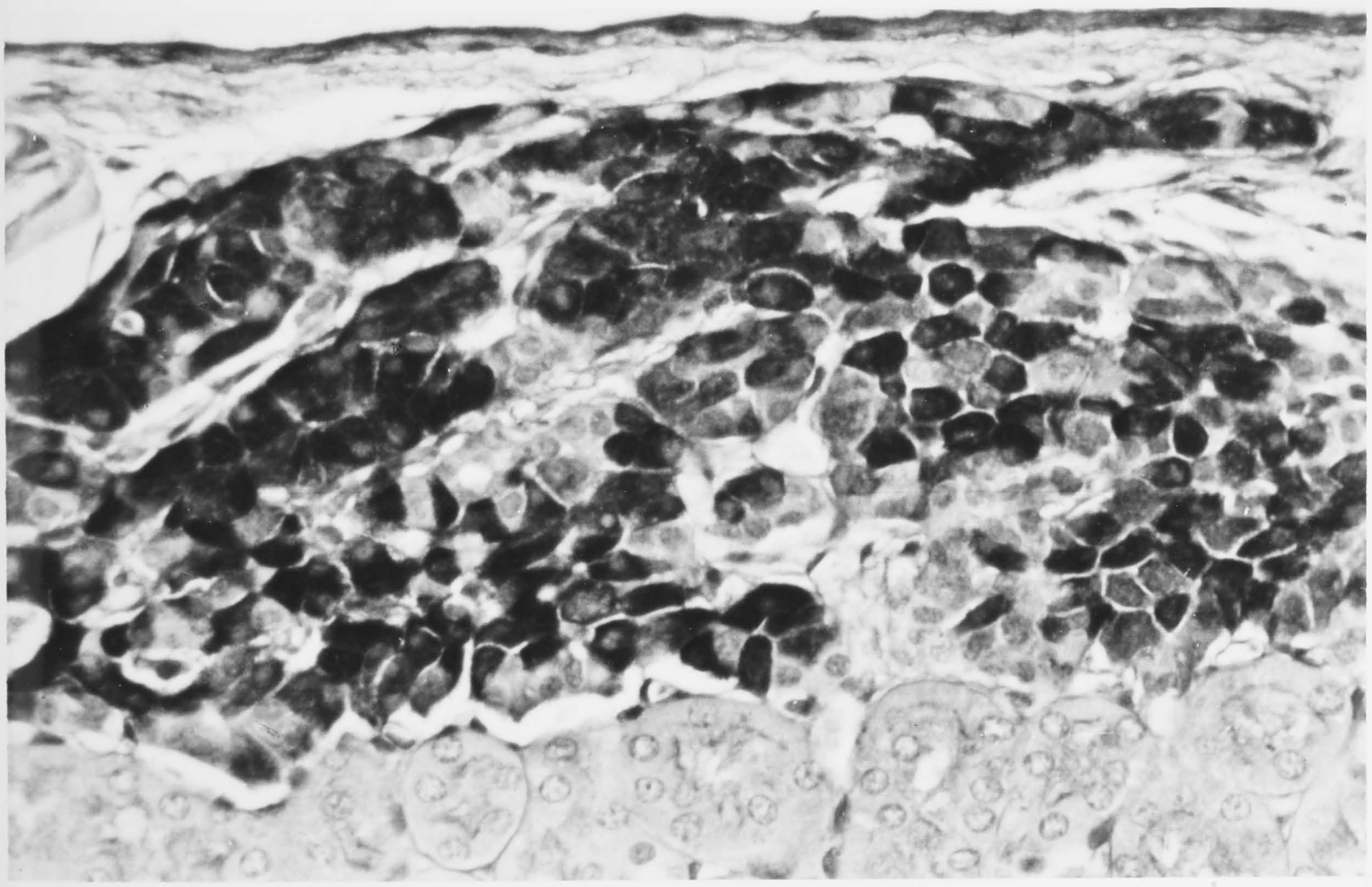
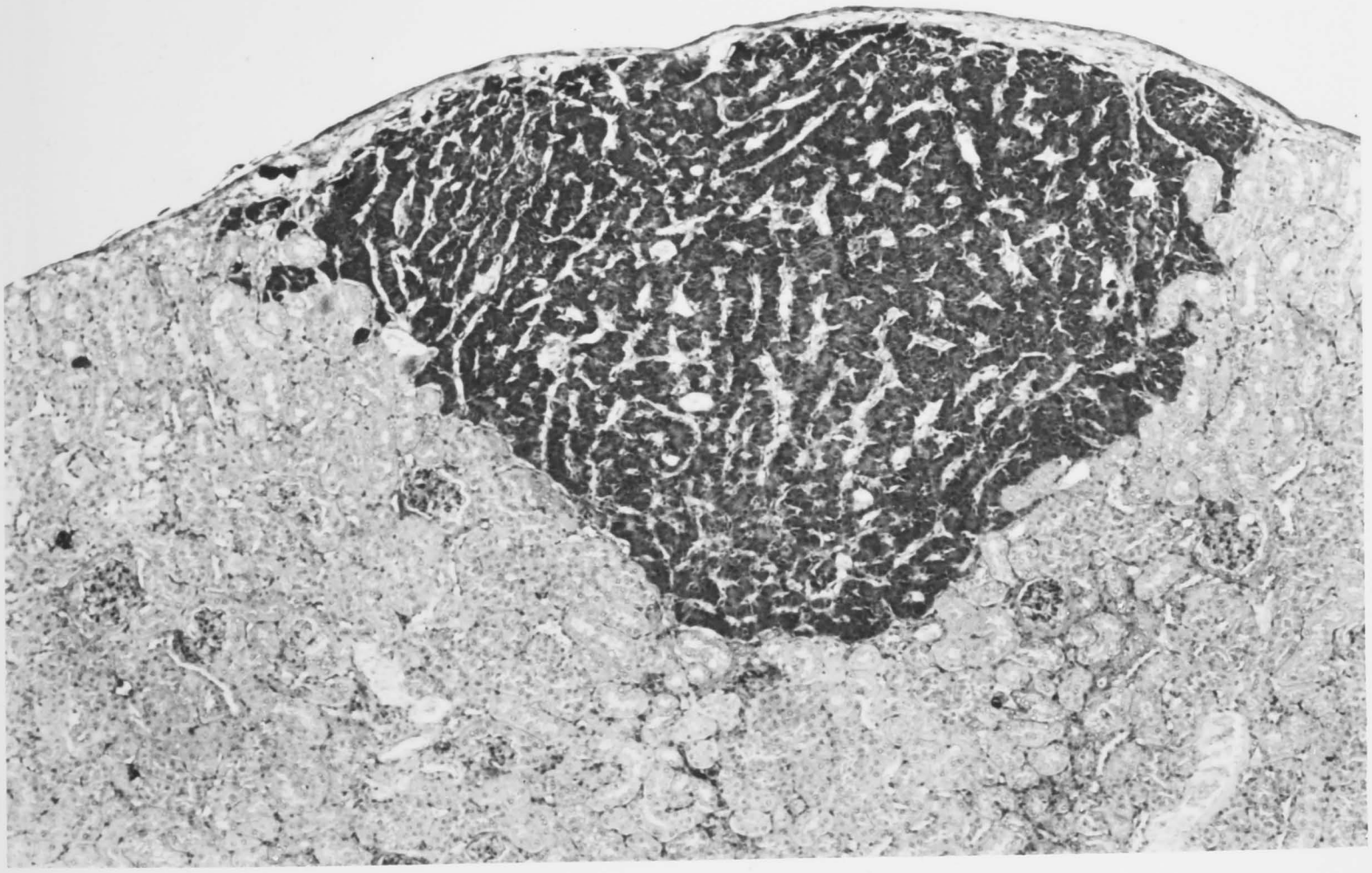


Figure 3.9 Host (CBA) response to allograft of islet tissue conditioned by organ culture of BALB/c islet tissue for 7 days before transplantation. Tissue was examined 14 days after transplantation.

(Top) haematoxylin and eosin (x 1370)

(Bottom) aldehyde-fuchsin (x 1370)



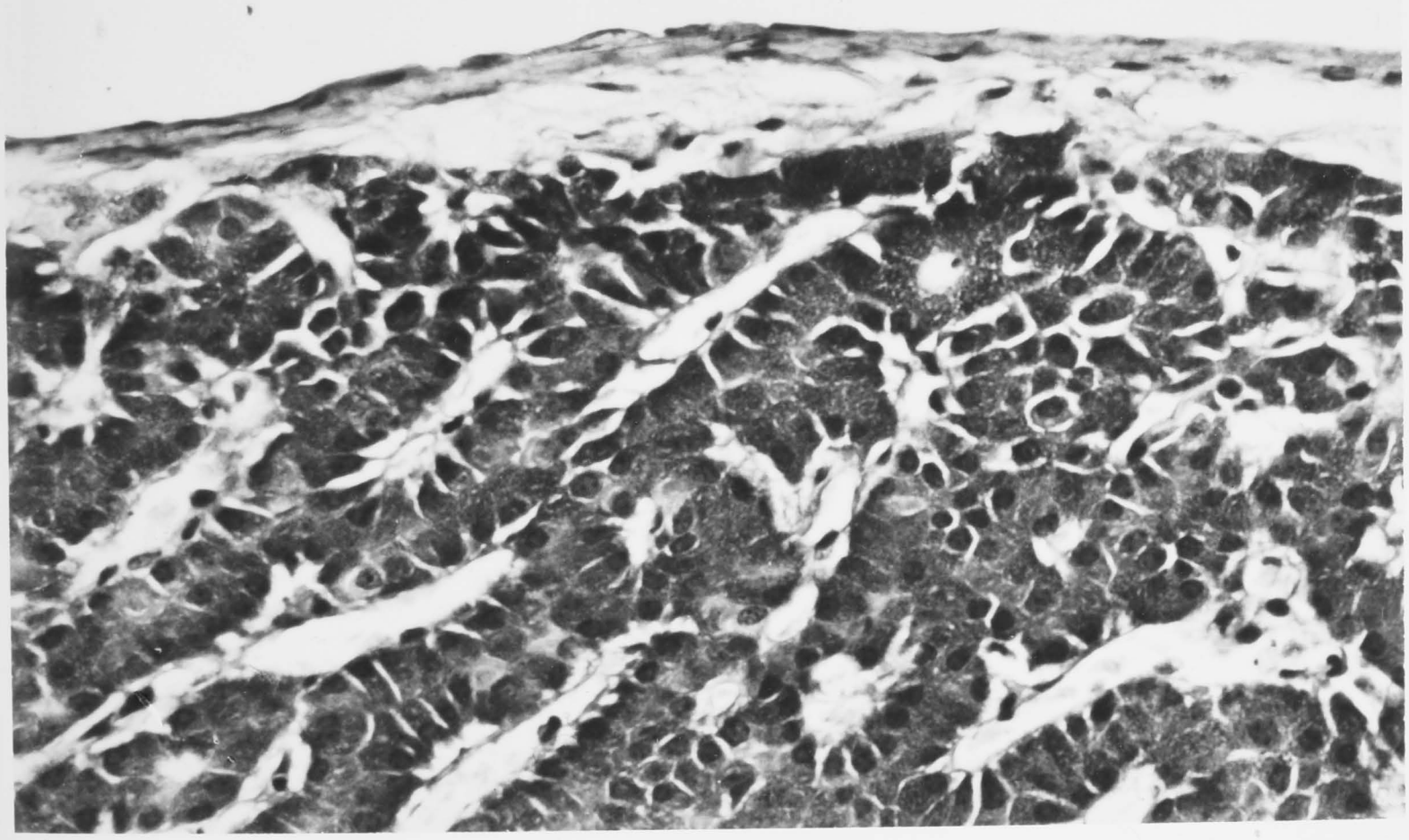


Figure 3.10 Islet cluster from a BALB/c donor transplanted under the renal capsule of a CBA recipient. The tissue was conditioned by a 7 day culture period, and the graft examined 420 days after transplantation. Note the absence of any allograft response and the extensive capillary network within the graft.

(Top) aldehyde-fuchsin (x 220)

(Bottom) aldehyde-fuchsin (x 1370)

Figure 3.11 Uncultured BALB/c pancreatic islet aggregates examined 28 days following transplantation into normal CBA recipients. The 4 grafts were either partially or completely rejected.

haematoxylin and eosin (x 900)

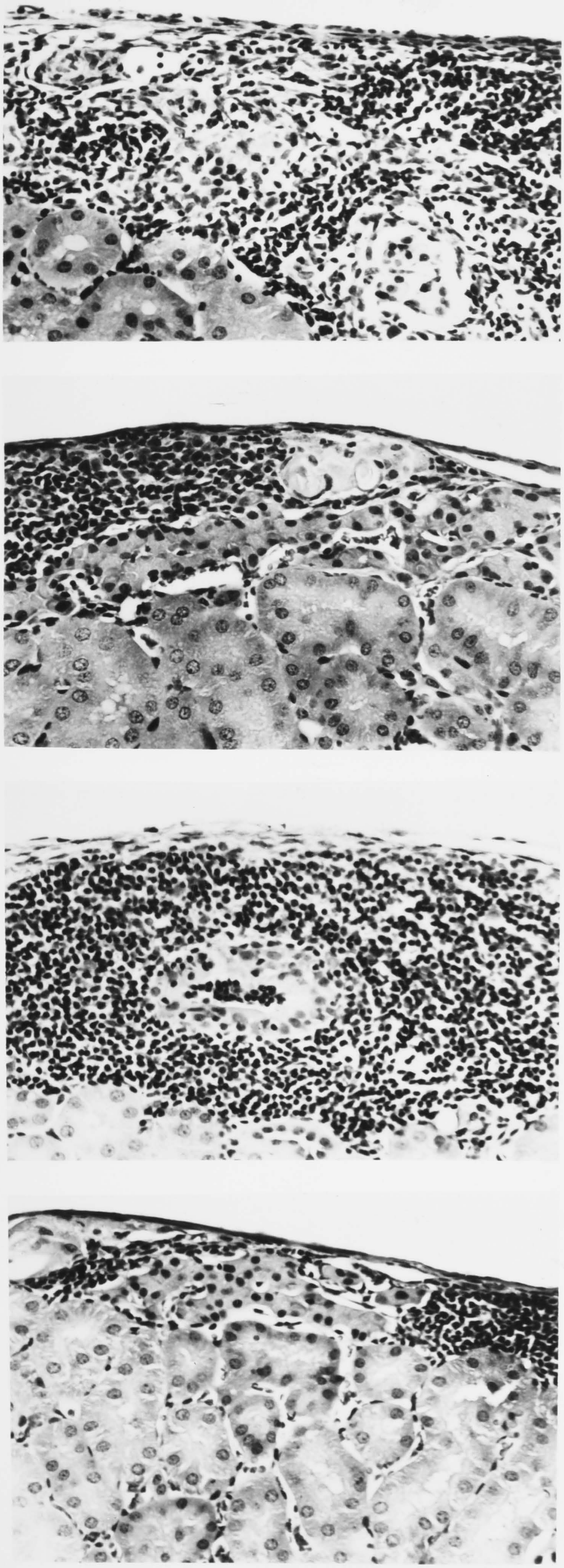


TABLE 3.4 Survival of BALB/c ($H\text{-}2^d$) islets aggregated for 4-6 hours or cultured for 7 days prior to transplantation to CBA ($H\text{-}2^k$) recipients

Duration of Culture (Days)	Time after transplantation (Days)	Histological Score: Mononuclear cell infiltration	Histological Score: Integrity of islets
0 (4-6 hours)	14	+	++++
		+++	+++
		++++	+
		++	++++
7	14	0	+++++
		+	+++++
		0	+++++
		0	+++++
0 (4-6 hours)	28	+++	++++
		++++	++
		++++	+
		++++	+
7	28	0	+++++
		0	+++++
		0	+++++
		0	+++++
0	420	not tested	
7	420	0	+++++
		0	+++++
		0	+++++
		0	+++++

Generality of Phenomenon

A further series of experiments was performed using donor-recipient strain combinations differing at various major and minor histocompatibility loci; these studies were carried out to determine whether the effect of culture on islet immunogenicity was a general phenomenon seen in different strain combinations, and to examine the effects of pancreatic islet culture on the immunogenicity of islets allografted across both major and minor histocompatibility barriers. In all of these experiments, cyclophosphamide pretreatment of the donors was withheld, as it had been shown to be unnecessary in the earlier experiments. The culture period was 7 days, and pancreatic islets aggregated in air and 5% CO_2 for 4 - 6 hours were used as day 0 control allografts.

Three donor-recipient strain combinations were tested, each with differences at the major histocompatibility (H-2) loci. BALB/c ($H\text{-}2^d$) mice were used as donors, and the 3 recipient strains were B10.G ($H\text{-}2^q$), A.TL ($H\text{-}2K^sI^kD^d$) and C57B1/6 ($H\text{-}2^b$). B10.G recipients of BALB/c pancreatic islets behaved in a similar way to CBA mice allografted with BALB/c islets, as a 7 day culture period significantly reduced graft immunogenicity with both these strain combinations. At 28 days following transplantation, the B10.G mice had rejected 7 of the 8 uncultured islet allografts, while in contrast, 7 of the 8 cultured islet allografts remained completely

intact (Table 3.5). With the BALB/c to C57Bl/6 strain combination, only 1 of the 10 day 0 control allografts was intact at 28 days. A 7 day period of organ culture significantly improved graft survival, with 6 out of 11 allografts surviving for 28 days (Table 3.6).

It is clear from the data so far presented that uncultured BALB/c islet aggregates rejected within 28 days when they were allografted to normal CBA, C57Bl/6, and B10.G recipients, and that when BALB/c islets were cultured in a 95% O_2 and 5% CO_2 gas phase for 7 days prior to transplantation to these recipient strains, allograft survival figures were significantly and consistently improved. However, when BALB/c islets were allografted to the A.TL mouse, 7 out of the 9 uncultured allografts showed no signs of rejection at 28 days, while all 11 of the cultured allografts were completely intact (Table 3.7). With this strain combination, it is evident that the uncultured islet allografts were generally poorly immunogenic and that a 7 day period of organ culture prior to transplantation is largely unnecessary. Uncultured BALB/c ($H\text{-}2^d$) islet aggregates were also shown to be poorly immunogenic when grafted across a minor histocompatibility barrier into DBA/2 ($H\text{-}2^d$) recipients, as 4 out of the 5 uncultured BALB/c islet aggregates were completely intact when removed from their DBA/2 hosts 28 days following transplant (Table 3.8). All 6 of the 7-day cultured islet clusters survived completely intact. Table 3.9 provides a summary of the overall results.

TABLE 3.5 Survival of BALB/c (H-2^d) islets aggregated for 4-6 hours or cultured for 7 days prior to transplantation to B10.G (H-2^q) recipients

Duration of Culture (Days)	Histological Score	
	Mononuclear cell infiltration	Integrity of islets
0	+	+++++
(4-6 hours in air)	+++	++
	0*	0
	0*	0
	0*	0
	0*	0
	0*	0
	0*	0
	0*	0
7	++	+++++
(95% O_2 + 5% CO_2)	+	+++++
	+	+++++
	+	+++++
	0	+++++
	0	+++++
	0	+++++
	+++	++

* Grafts not examined histologically, as only scar tissue was present when grafts were examined macroscopically.

TABLE 3.6 Survival of BALB/c ($H\text{-}2^d$) islets aggregated for 4-6 hours or cultured for 7 days prior to transplantation to C57B1/6 ($H\text{-}2^b$) recipients. Grafts were examined 28 days following transplantation.

Duration of Culture (Days)	Histological Score	
	Mononuclear cell infiltration	Integrity of islets
0	0	+++++
(4-6 hours in air)	++	++++
	++++	+
	++++	+
	++++	0
	+	0
	+	0
	0*	0
	0*	0
	0*	0
7	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++
	+	+++++
	+++	+++
	+++	++
	+++	++
	+	0
	0*	0

* Scar tissue only, with no signs of mononuclear cell infiltration

TABLE 3.7 Survival of BALB/c ($H\text{-}2^d$) islets aggregated for 4-6 hours or cultured for 7 days prior to transplantation to A.TL ($h\text{-}2K^sI^kD^d$) recipients.

Duration of Culture (Days)	Histological Score	
	Mononuclear cell infiltration	Integrity of islets
0 (4-6 hours in air)	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++
	+	+++++
	+	+++++
	++	++++
	0*	0
7	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++
	+	+++++

* Scar tissue only, with no signs of mononuclear cell infiltration

TABLE 3.8 Survival of BALB/c ($H\text{-}2^d$) islets aggregated for 4-6 hours or cultured for 7 days prior to transplantation to DBA ($H\text{-}2^d$) recipients. Grafts examined 28 days following transplantation.

Duration of Culture (Days)	Histological Score	
	Mononuclear cell infiltration	Integrity of islets
0	+++	+++
(4-6 hours in air)	0	+++++
	0	+++++
	0	+++++
	0	+++++
7	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++
	0	+++++

TABLE 3.9 Comparison of the effect of major and minor histocompatibility differences on the survival of day 0 islet aggregates and 7 day cultured islet clusters.

Donor	Recipient	Proportion of allografts completely intact 28 days post-transplant	
		Day 0 aggregate	Day 7 cluster
BALB/c ($H\text{-}2K^dI^dD^d$)	A.TL ($H\text{-}2K^sI^kD^d$)	7/9	11/11
	CBA ($H\text{-}2K^kI^kD^k$)	0/4	9/9*
	B.10G ($H\text{-}2K^qI^qD^q$)	1/9	7/8
	C57B1/6 ($H\text{-}2K^bI^bD^b$)	1/10	6/11
BALB/c ($H\text{-}2K^dI^dD^d$)	DBA ($H\text{-}2K^dI^dD^d$)	4/5	6/6

(Minor histocompatibility differences only)

* 5/5 intact at 420 days

DISCUSSION

Available data show that pancreatic islets are highly immunogenic, with normal pancreatic islets generally evoking a vigorous and rapid allograft response. Garvey *et al* (1979) have reported that it is more difficult to suppress an immune response to transplanted islets than a response to either kidney or skin allografts, while Barker and his colleagues (Reckard & Barker 1973; Frangipane, Barker & Silvers 1977) have produced data showing that functional rejection of rat and mouse islets occurs within 7 days when grafted across a minor histocompatibility barrier. They have also shown that with the C57Bl/6 strain a H-Y difference is sufficient to produce rapid graft rejection, with male C57Bl/6 islets rejecting within 7 days of grafting to C57Bl/6 recipients. In contrast male C57Bl/6 skin grafted to female C57Bl/6 recipients survived for up to 29 days. However, the experimental data presented here show that the immunogenicity of pancreatic islets can be reduced or eliminated by treatment of the islet tissue before transplantation. The treatment procedures were based on Lafferty's previous experience with thyroid allotransplantation, which showed that organ culture reduced graft immunogenicity, and indicated that leucocytes carried within the graft were likely to be the major barrier to successful allotransplantation (Talmage *et al* 1976; Lafferty & Woolnough 1977).

The bulk of this study was carried out using the BALB/c($H\text{-}2^d$) to CBA($H\text{-}2^k$) donor-recipient strain combination, as most of

the thyroid allograft work had been performed with this combination (Lafferty & Woolnough 1977). With uncultured thyroid allografts, cyclophosphamide pretreatment of the tissue donor produced a more chronic rejection process, and this situation also applied to uncultured BALB/c islet allografts taken from pretreated donors. When cyclophosphamide pretreatment of the donor was combined with organ culture, the thyroid (Lafferty & Woolnough 1977) and islets again behaved in similar fashion, with little or no immune response to the allografted tissue being detected. Although very small pockets of lymphoid cells were sometimes seen around the islet tissue 14 days after transplantation, islet damage due to an allograft response was seen in only 1 of the 38 transplants examined. All other allografts had normal histology, and contained obvious beta cells when stained with aldehyde-fuchsin. The minor mononuclear cell response appeared to be a transient phenomenon because none of the grafts examined 84 and 420 days after transplantation showed any trace of an allograft response.

Although others have attempted to condition islets for allotransplantation by means of tissue culture, all used a gas phase of air, and had little success in prolonging islet allograft survival by use of this technique alone (Andersson & Buschard 1977; Kedinger *et al* 1977; Lacy, Davie & Finke 1979b). Talmage & Dart (1978) reported that the oxygen tension of the gas phase plays an important role in conditioning thyroid tissue for allotransplantation, presumably because of its

selective toxicity for blood cells carried within the transplanted tissue, and the method of pancreatic islet culture outlined in this thesis was designed to take advantage of this effect. Nevertheless, Lacy, Davie & Finke (1979a) have shown that the culture of rat pancreatic islets in air at an ambient temperature of 24^{o} for 7 days prior to transplantation to allogeneic recipients enabled reversal of streptozotocin diabetes for longer than 200 days, provided the recipient had one treatment with antilymphocyte serum (ALS) at the time of islet transplantation. This group reported that culture alone for 21 days prior to transplant gave a slight but statistically significant increase in duration of graft survival over uncultured control islets (7.2 to 11.6 days mean survival time), whereas ALS given at the time of transplantation of uncultured islets prolonged allograft survival to a mean survival time of 61 days (Lacy, Davie & Finke, 1979b). The affects of culture in air plus ALS treatment of the graft recipient were thus synergistic, with combined treatment being necessary for long-term survival of the majority of the allografts.

In the present study, it is clear that cultured BALB/c islets will survive for up to 420 days after grafting to normal CBA recipients, and that cyclophosphamide pretreatment of the donor was not needed as an adjunct in reducing allograft immunogenicity. These favourable results suggest that the high oxygen gas phase used in the culture system plays a crucial role in the conditioning of islet tissue

for allotransplantation. Testing of a further 3 strain combinations with differences at the major histocompatibility complex showed that the ease of conditioning mouse pancreatic islets for transplantation may depend on the strain combination used, as a 7 day period of organ culture was not sufficient to ensure 100% survival of BALB/c islet clusters allografted to C57Bl/6 recipients, even though the results were significantly better than those obtained with day 0 allografts. On the other hand, organ culture was shown to be superfluous when the BALB/c to A.TL strain combination was used. Here, the donor and recipient strains differ at the K and I regions of the MHC, and the result thereby shows that genetic differences at the MHC do not inevitably result in the rejection of uncultured islet allografts. Although there is no clear-cut explanation for this result, it is possible that it is related to donor-recipient histocompatibility at the H-2D region of the MHC, although data available for other tissues shows that it is H-2K compatibility between donor and recipient strains that generally correlates with a reduction in graft immunogenicity (Klein 1975). Uncultured BALB/c islets grafted across a minor histocompatibility barrier into DBA/2 recipients proved to be poorly immunogenic, and uncultured thyroid allografts between these two strains have recently been shown to behave in similar fashion, with grafts remaining functional for at least 250 days (Simeonovic - personal communication). In contrast, skin grafts from BALB/c donors to DBA/2 recipients are rejected

within 20 - 30 days of transplantation (Prehn & Main 1954; Simeonovic - personal communication). At present, the cause(s) for these differences in graft immunogenicity are not understood, and require further investigation.

There are several ways by which organ culture might affect graft immunogenicity. The role of donor stimulator leucocytes has been extensively explored with Lafferty's thyroid allograft model (Lafferty & Woolnough 1977), with all the evidence suggesting that high oxygen tissue culture prior to allotransplantation selectively inactivates stimulator leucocytes within the graft, and that this loss of stimulatory activity renders the allograft non-immunogenic. In addition, they observed loss of vascular endothelium from cultured mouse thyroids, and this also occurs with the cultured pancreatic islet (Chapter 6). Islet capillary endothelium carries H-2 antigen (Parr 1979b) and Hirschberg *et al* (1975) have claimed that vascular endothelial cells have an intrinsic stimulatory capacity. If this were so, destruction of vascular endothelium during organ culture would further reduce donor stimulatory capacity as well as altering the antigenicity of the graft. However, it is unlikely that vascular endothelium plays a major role in the generation of an immune response as Lafferty & Woolnough (1977) showed that cyclophosphamide pretreatment of thyroid allograft donors did not destroy donor-strain endothelium prior to transplantation. Despite this, 30% of these grafts survived

for prolonged periods in normal hosts.

Further evidence that vascular endothelium *per se* is not likely to be high immunogenic *in vivo* was recently provided by Batchelor and his colleagues (1979). These workers allografted (AS X AUG) F_1 rat kidneys into passively enhanced AS recipients, and after 30 - 90 days functional allografts were re-transplanted into naive AS secondary recipients. These animals accepted the re-transplanted (AS X AUG) F_1 kidneys for a prolonged period, with 4 of the 10 grafts surviving for greater than 100 days. In contrast, control animals (naive AS rats receiving normal F_1 kidneys) rejected their allografts within 12 days. When 6 secondary graft recipients were sensitised to donor antigen prior to receiving a re-transplanted kidney, hyperacute rejection of the grafts occurred in 5 out of the 6 rats. Hyperacute rejection, which is primarily antibody mediated, is directed against vascular endothelium (Kissmeyer-Nielsen *et al* 1966), and the latter experiment thereby showed that the vascular endothelium of the donor was not replaced by that of the host in long-surviving passively-enhanced renal allografts. Furthermore, this experiment showed that donor endothelium still carried detectable transplantation antigen, despite its demonstrated lack of capacity to stimulate graft rejection, in secondary naive recipients. This finding in the rat has been confirmed by Hart, Winearls & Fabre (1980), and the conclusion of both groups has been that the development of tolerance in the secondary host was due to the loss of stimulator leucocytes

from the kidney during the time it remains grafted to the enhanced primary host (Welsh *et al* 1979).

Batchelor *et al* (1979) consider that the presentation of graft antigen in a non-immunogenic form induces a selective tolerance in the naive secondary recipient. They had previously shown that major histocompatibility complex antigens act as strong immunogens only when presented to the host as surface antigens on viable leucocytes, and that membrane preparations containing similar quantities of antigen fail to produce either a cell-mediated or humoral response (Batchelor, Welsh & Burgos 1978). The 2 signal models for lymphocyte activation proposed by Bretscher & Cohn (1970) and Lafferty & Cunningham (1975) predict that presentation of antigen in a non-immunogenic form will lead to the development of selective tolerance, and Batchelor's experimental findings are generally consistent with this hypothesis (Welsh, Burgos & Batchelor 1977; Batchelor, Welsh & Burgos 1978; Batchelor *et al* 1979; Welsh *et al* 1979). This question is further examined in Chapter 5 of this thesis.

In summary, the experimental results show that organ culture of mouse pancreatic islets in a 95% oxygen gas phase has the effect of either reducing or eliminating their immunogenicity and suggest that the application of similar techniques to reduce the immunogenicity of human pancreatic islet tissue prior to allotransplantation into diabetic recipients should be investigated.

CHAPTER FOUR

REVERSAL OF STREPTOZOTOCIN-DIABETES WITH CULTURED ISLET ALLOGRAFTS

CHAPTER FOUR

INTRODUCTION

The histological studies detailed in Chapter 3 demonstrate that cultured BALB/c pancreatic islets will survive indefinitely when grafted across a major histocompatibility barrier into normal, non-immunosuppressed CBA recipients. These data provide no direct evidence of graft function; if a graft is to be so termed, it must provide either partial or complete restoration of normoglycaemia (euglycaemia) to a previously diabetic animal, with the degree of reversal being dependent on the mass of functional tissue grafted. Although 600-1400 isolated islets are necessary for reversal of streptozotocin-diabetes in the rat (Gray & Watkins 1976; Nash, Peters & Bell 1978; Lacy, Davie & Finke 1979a and b), isograft studies have shown that 300-600 isolated islets will reverse streptozotocin-diabetes in the mouse (Andrus - personal communication; Frangipane, Barker & Silvers 1977). The experiments that follow were designed to test the proposition that 300 to 350 cultured mouse pancreatic islets had the functional capacity sufficient to reverse streptozotocin-diabetes in allogeneic recipients.

RESULTS

A preliminary experiment was performed to assess the effect of 6 BALB/c islet clusters (approximately 300 islets) grafted into diabetic CBA recipients. Six diabetic CBA mice were divided into 3 groups of 2 mice. One group was transplanted with BALB/c islets aggregated for 4 hours in 5% CO_2 and air, the second group received 7-day cultured islet tissue, and the third group received no grafts. Non-fasting blood sugar levels were determined on each animal just before transplantation, and repeated a number of times thereafter.

Figure 4.1 shows the data obtained from the 2 animals in each group. Within 7 to 10 days of transplantation, the blood sugar levels of animals transplanted with both cultured and uncultured islets returned to the normal range. By 14 - 21 days post-transplantation rejection of the uncultured islets commenced and the blood sugar levels of these animals returned to the same level as those of the untreated controls. Animals receiving cultured islet allografts maintained their non-fasting blood sugar levels either within the normal range or within 4mmol/l of the upper limit of the normal range (8.6 ± 1.2mmol/l).

The 6 animals lost between 13 and 29% of their initial body weight by 14 days after the induction of diabetes. Those that received cultured allografts regained their pre-morbid body weight by 60 days after transplantation. Animals grafted with uncultured allografts showed initial

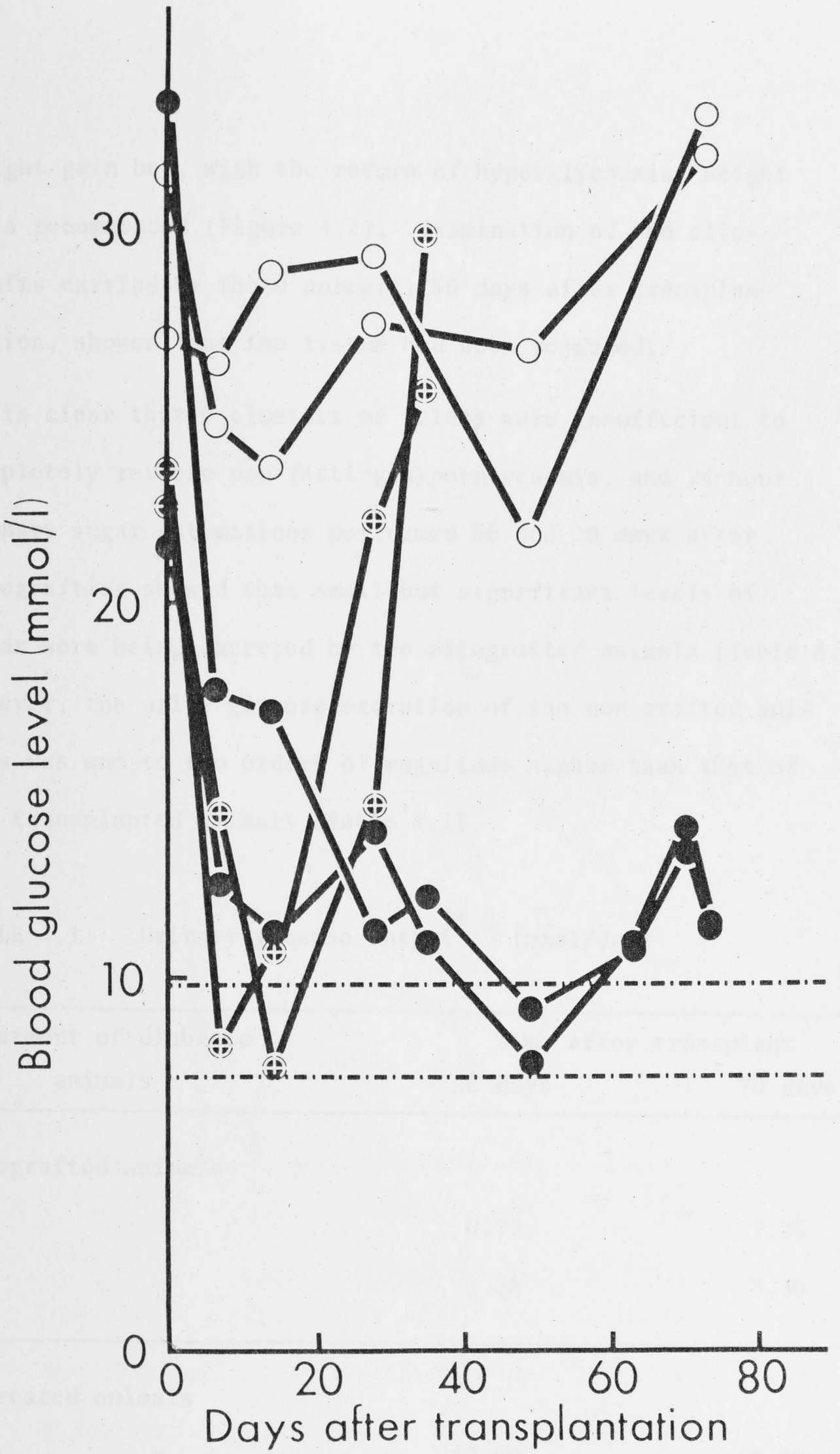


Figure 4.1 Non-fasting blood glucose levels of CBA mice after induction of diabetes

⊕ animals allografted with 300 uncultured BALB/c islets

● animals allografted with 300 cultured BALB/c islets

○ control animals - no graft

(the dotted lines represent the 95% confidence interval for non-fasting blood glucose levels in normal CBA mice)

weight gain but, with the return of hyperglycaemia, weight loss recommenced (Figure 4.2). Examination of the allografts carried by these animals, 50 days after transplantation, showed that the tissue had been rejected.

It is clear that 6 clusters of islets were insufficient to completely reverse non-fasting hyperglycaemia, and 24 hour urinary sugar estimations performed 56 and 70 days after allografting showed that small but significant levels of sugar were being excreted by the allografted animals (Table 4.1). However, the urine glucose excretion of the non-grafted animals was one to two orders of magnitude higher than that of the transplanted animals (Table 4.1).

TABLE 4.1 Urinary glucose output[1] (mmol/day)

Treatment of diabetic animals	Time after transplant 56 days	70 days
Allografted animals		
(1)	0.72	1.25
(2)	0.36	0.36
Untreated animals		
(1)	17.60	17.20
(2)	15.00	17.20

[1] Normal animals excrete less than 0.2mmol glucose/day

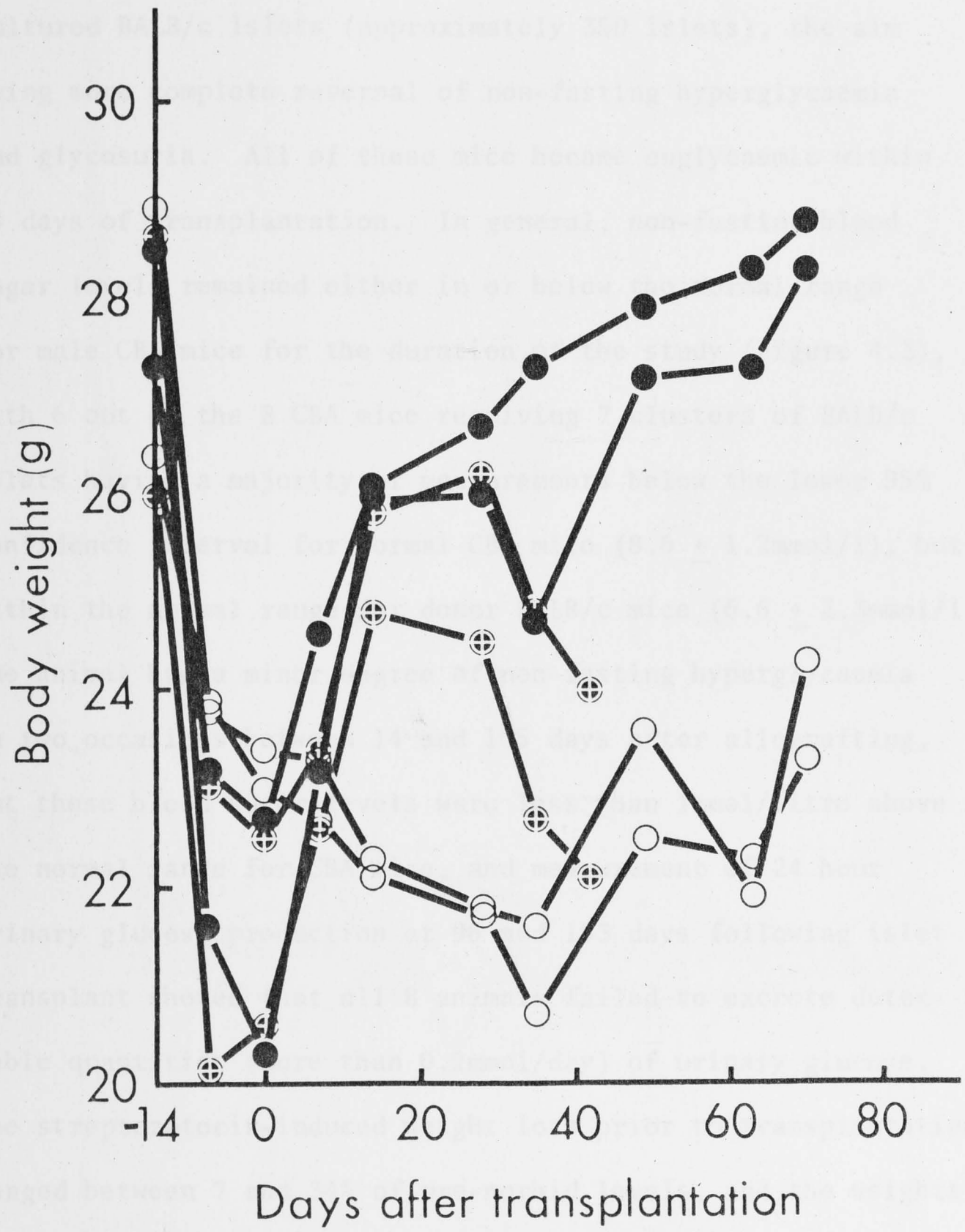


Figure 4.2 Body weights of streptozotocin-diabetic CBA mice

⊕ animals allografted with 300 uncultured BALB/c islets

● animals allografted with 300 cultured BALB/c islets

○ non-grafted control animals

A further 8 diabetic CBA mice were then grafted with 7-day cultured BALB/c islets (approximately 350 islets), the aim being more complete reversal of non-fasting hyperglycaemia and glycosuria. All of these mice became euglycaemic within 14 days of transplantation. In general, non-fasting blood sugar levels remained either in or below the normal range for male CBA mice for the duration of the study (Figure 4.3), with 6 out of the 8 CBA mice receiving 7 clusters of BALB/c islets having a majority of measurements below the lower 95% confidence interval for normal CBA mice (8.6 $\pm$ 1.2mmol/l), but within the normal range for donor BALB/c mice (6.6 $\pm$ 2.3mmol/l). One animal had a minor degree of non-fasting hyperglycaemia on two occasions between 14 and 105 days after allografting, but these blood sugar levels were less than 1mmol/litre above the normal range for CBA mice, and measurement of 24 hour urinary glucose production at 96 and 103 days following islet transplant showed that all 8 animals failed to excrete detectable quantities (more than 0.2mmol/day) of urinary glucose. The streptozotocin-induced weight loss prior to transplantation ranged between 7 and 34% of pre-morbid levels, and the weights of all 8 animals had returned to these initial levels by 91 days following islet transplantation.

These results suggest that 350, rather than 300 cultured BALB/c(H-2^d) mouse islets is likely to be the optimal number required for successful reversal of streptozotocin-induced hyperglycaemia in allogeneic CBA(H-2^k) transplant recipients.

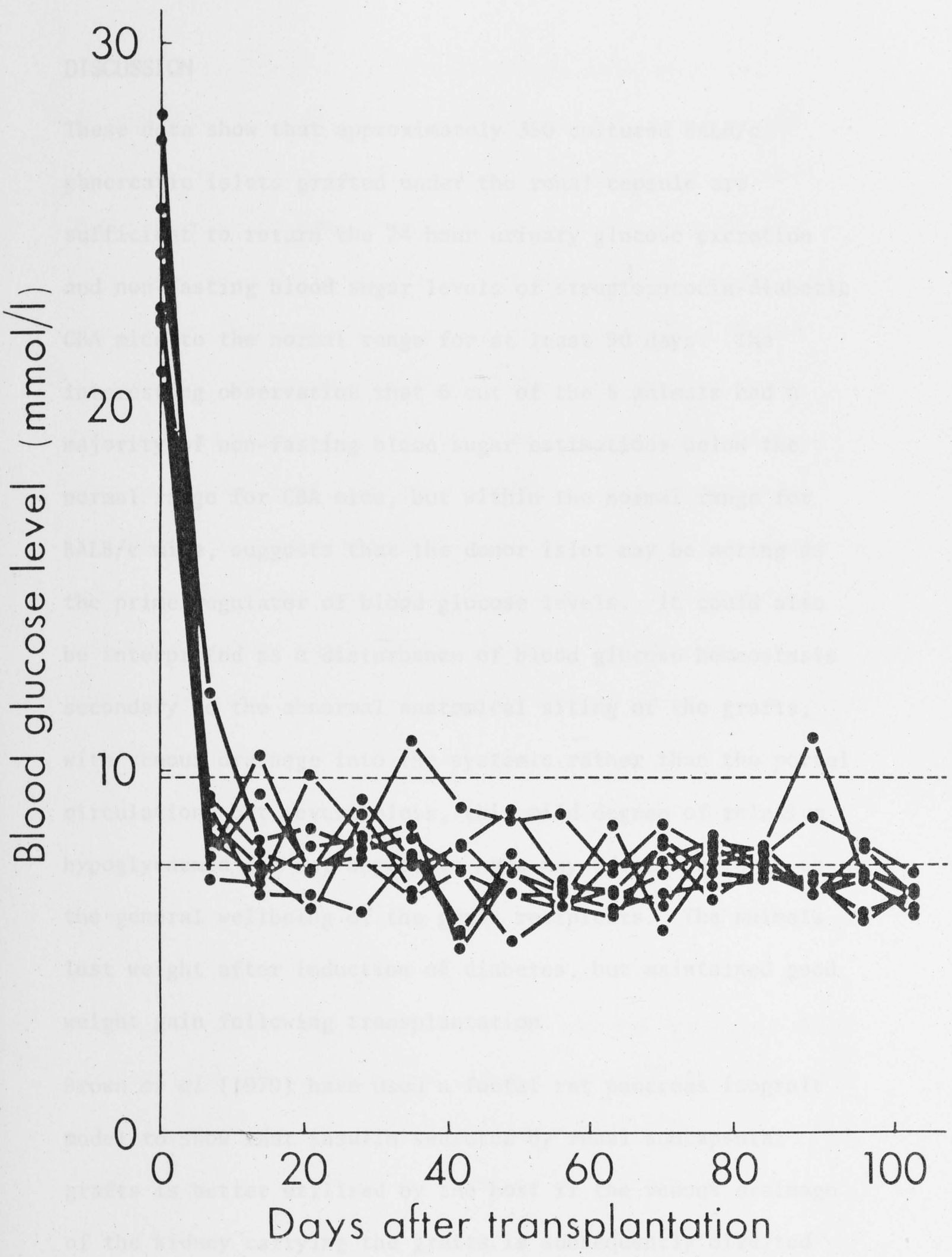


Figure 4.3 Non-fasting blood glucose levels of 8 diabetic CBA mice grafted with 350 cultured BALB/c islets.

(The dotted lines represent the 95% confidence interval for non-fasting blood glucose levels in normal CBA mice)

DISCUSSION

These data show that approximately 350 cultured BALB/c pancreatic islets grafted under the renal capsule are sufficient to return the 24 hour urinary glucose excretion and non-fasting blood sugar levels of streptozotocin-diabetic CBA mice to the normal range for at least 90 days. The interesting observation that 6 out of the 8 animals had a majority of non-fasting blood sugar estimations below the normal range for CBA mice, but within the normal range for BALB/c mice, suggests that the donor islet may be acting as the prime regulator of blood glucose levels. It could also be interpreted as a disturbance of blood glucose homeostasis secondary to the abnormal anatomical siting of the grafts, with venous drainage into the systemic rather than the portal circulation, but nevertheless, this mild degree of relative hypoglycaemia did not appear to have any adverse affects on the general wellbeing of the graft recipients. The animals lost weight after induction of diabetes, but maintained good weight gain following transplantation.

Brown *et al* (1979) have used a foetal rat pancreas isograft model to show that insulin secreted by renal subcapsular grafts is better utilised by the host if the venous drainage of the kidney carrying the grafts is subsequently diverted to the portal circulation. To do this, they partially reversed experimental diabetes in rats by grafting 2-3 syngeneic foetal pancreata under the renal capsule, and then demonstrated

the rapid restoration of normal glucose tolerance, and return of 24 hour urinary glucose excretion to the normal range, following the establishment of a hepato-renal shunt. Their findings are important, but do not rule out the use of transplantation sites drained by the systemic venous circulation, for Brown's group has previously shown (Brown *et al* 1976) that syngeneic foetal pancreata grafted under the renal capsule would restore normal glucose tolerance provided that 4 pancreata were grafted. More recently, Simeonovic (personal communication) has shown that a single mouse foetal pancreas isograft is sufficient to completely reverse experimental diabetes, provided that the graft recipients are treated with insulin for the first 40-60 days following transplantation.

These results show that the renal subcapsular site is satisfactory for endocrine pancreas transplantation, provided that optimal amounts of tissue are grafted. This site offers many technical advantages over the portal route, since grafts are easily positioned, can be readily exposed for observation *in vivo*, and can be recovered without the need to remove a vital organ. The risk of portal hypertension (Du Toit *et al* 1980) following injection of tissue via the portal vein is also avoided.

The present study shows that the renal subcapsular site can be successfully used for the type of islet transplantation study requiring both continual verification of allograft

function, and graft assessibility. Although formal glucose tolerance tests were not performed, and the kinetics of insulin release not examined, the restoration of non-fasting euglycaemia and undetectable urinary glucose excretion suggested adequate insulin secretion by all the allografts.

These results have clinical implications, since arrest or reversal of the complications seen in experimental diabetes has already been demonstrated by transplantation of isogeneic pancreatic islets (Mauer *et al* 1974; Krupin *et al* 1979). Gray & Watkins (1976) have shown that allotransplantation of rat islets across a minor histocompatibility barrier into chronically immunosuppressed recipients will prevent the renal and retinal complications of experimental diabetes, and it is reasonable to assume that islet allografts pre-conditioned by organ culture will have a similar beneficial effect on the complications of experimental diabetes. Furthermore, the successful adaptation of this technique to clinical islet transplantation may reduce the risk of prolonged immunosuppressive therapy compromising the patient's resistance to opportunistic infection (Hall *et al* 1976; Toussaint *et al* 1978), but before clinical islet allotrans-plantation becomes a realistic proposition, the problems involved in obtaining sufficient human islet tissue need to be overcome (Scharp *et al* 1980). However, pancreatic islet xenografts between closely related species (rat to mouse) have already been shown to markedly prolong graft survival

if the donor islets are cultured in air and 5% CO_2 for 7 days prior to transplantation, and the recipient is given a single injection of either mouse antilymphocyte serum (ALS) or mouse plus rat ALS (Lacy, Davie & Finke 1980). Suitable adaption of an islet xenograft procedure for the clinical treatment of diabetes mellitus may partially solve the problem of obtaining donor tissue, as a closely related species such as the monkey, could become a source. Eloy and her colleagues (1979) have claimed that uncultured embryonic chick pancreas will produce a partial but sustained reversal of experimental hyperglycaemia in rats. They have suggested that 15 day embryonic chick pancreas is incapable of stimulating a host immune response, and if this should prove to be correct, then use of embryonic chick pancreas may also have potential for clinical transplantation. All of these encouraging findings provide justification for persevering with attempts to cure human diabetes mellitus by means of islet transplantation.

CHAPTER FIVE

EFFECTS OF INJECTION OF DONOR LEUCOCYTES ON THE SURVIVAL OF PANCREATIC ISLET ALLOGRAFTS

CHAPTER FIVE

INTRODUCTION

It is now possible to reduce the immunogenicity of a number of rodent endocrine organs by maintaining them in tissue culture prior to transplantation across major histocompatibility barriers. The technique has been shown to prolong the survival of allografted mouse thyroid, parathyroid and ovary (Jacobs 1974; Lafferty *et al* 1976a) mouse pancreatic islets (Chapter 3) and rat parathyroid and pancreatic islets (Naji, Silvers & Barker 1979; Lacy, Davie & Finke 1979b). Optimal results have been achieved when the organs are cultured in a 95% O_2 gas phase (Lafferty 1975; Chapter 3 of this thesis) with 100% long-term (greater than 420 day) survival of mouse pancreatic islets subsequently allografted to non-immunosuppressed recipients. With the thyroid, it is likely that organ culture destroys donor-strain stimulator leucocytes, as stimulation of the host immune response to a cultured thyroid allograft can be achieved if 10^5 donor-strain peritoneal cells are injected intravenously at the time of transplantation (Lafferty *et al* 1976b), showing that the cultured thyroid allografts retained recognisable transplantation antigen.

It is important to know whether cultured pancreatic islet allografts carry recognisable transplantation antigen at the time of allografting, and also to determine whether a host immune response can still be elicited after islet allografts have remained *in situ* for a prolonged period of time. Theoretical models (Bretscher & Cohn 1970; Lafferty & Cunningham 1975) predict that exposure of lymphocytes to antigen alone may induce a state of host tolerance. These questions are examined in this study.

It is also important to determine whether a non-specific stimulus to the immune system is capable of inducing the rejection of cultured islet allografts. *In vitro* experiments have shown that a soluble mediating factor (lymphokine, monokine) obtained from a population of spleen cells stimulated with the T cell mitogen Concanavalin A will provide a source of a non-specific second signal, required for the antigen specific activation of cytotoxic T cells (Lafferty & Woolnough 1977). *In vivo*, any agent that can stimulate endogenous production of soluble mediating factors in the host subsequent to allotransplantation could conceivably lead to the activation of the sub population of T-lymphocytes bearing receptors for graft allo-antigen. Lafferty *et al* (1976a) indirectly examined the likelihood of a non-specific stimulus causing graft rejection when they attempted to induce the rejection of established cultured BALB/c($H\text{-}2^{d}$) thyroid allografts with a graft of an uncultured C57B1/6($H\text{-}2^{b}$) thyroid allograft to

the contralateral kidney of CBA(H-2^k) recipients. In this situation, the uncultured C57B1/6 allograft was promptly rejected, while the cultured BALB/c allograft remained intact. This data suggests that the effect of any soluble mediating factors released by donor-strain stimulator leucocytes carried within the uncultured C57B1/6 thyroid allograft was not sufficient to trigger a host response to allo-antigen carried on the cultured BALB/c thyroid. Nevertheless, a number of bacterial products are known to amplify immune responses *in vivo* and *in vitro* (Wahl, Wahl & McCarthy 1980). These adjuvants include lipopolysaccharides (LPS) and cell wall components of mycobacteria. In particular, the mycobacterial component of Freunds complete adjuvant is known to enhance T cell and macrophage cell activity (Allison & Davies 1971; Modelell *et al* 1974; Lowy, Bona & Chedid 1979), and to stimulate the release from macrophages of soluble factors such as lymphocyte activating factor (Interleukin I) (Togawa *et al* 1978). The effects of such non-specific activation of the host immune response mechanisms on survival of long-term functional mouse pancreatic islet allografts is also examined in this study.

It was found that cultured BALB/c pancreatic islet allografts retained transplantation antigen, but did not induce a state of tolerance in normal CBA recipients. In contrast, the results of functional studies suggested that 50% of the animals tested had developed hyporesponsiveness to donor-strain

transplantation antigen. A non-specific challenge to the host immune system failed to generate any detectable allograft response.

RESULTS

Survival of cultured islet allografts after challenge of the host with donor-strain peritoneal cells injected at the time of transplantation

In order to determine whether cultured pancreatic islet allografts carry recognisable transplantation antigen at the time of grafting, 10 normal CBA mice were each grafted with a single cluster (approximately 50 islets) of BALB/c islets previously cultured for 7 days. The animals were then immediately challenged with 10^5 peritoneal cells injected intravenously. The 5 test animals received 10^5 donor-strain (BALB/c) peritoneal cells, while the 5 control animals were injected with host strain (CBA) peritoneal cells. At 28 days following transplantation, the kidneys bearing the allografted islet clusters were removed for macroscopic and histological examination (Table 5.1). It was found that all 5 of the mice challenged with donor-syngeneic (BALB/c) peritoneal cells had rejected their allografts. The 5 control animals that had received host strain (CBA) peritoneal cells showed no signs of graft rejection. These results show that cultured BALB/c islet allografts are non-immunogenic at the time of transplantation, but retain

transplantation antigen that is recognised by the host animal following activation of the recipient's immune system by the injection of donor-strain leucocytes.

Survival of cultured islet allografts after challenge of the host with donor-strain peritoneal cells injected 360 days following transplantation

The data presented in the previous section demonstrated that, despite their lack of immunogenicity, cultured mouse pancreatic islets carry transplantation antigen at the time of allografting. Presumably, long surviving cultured islet allografts continue to retain recognisable antigen, albeit in a non-immunogenic form. A challenge with donor-strain peritoneal cells would confirm this, provided that the host had not become tolerised by the allograft. Accordingly, CBA mice that had received cultured BALB/c islet allografts 330 days beforehand underwent laparatomy under Avertin anaesthesia, and the integrity of the allografts assessed by visual examination. A group of 6 animals with intact allografts were then challenged 30 days later with 10^5 donor-strain peritoneal cells (p.c.) while a further 4 animals were kept as untreated controls. At 28 days after the injection of the cells all the animals were killed, and their grafts examined both macroscopically and histologically. The injection of 10^5 donor syngeneic (BALB/c) cells resulted in the total rejection of all 6 allografts, and examination of the control group showed 3 out of 4 allografts to be intact

(Table 5.1). It is likely that the remaining control allograft was lost within extensive adhesions.

TABLE 5.1 Pancreatic islet allograft survival after challenge with 10^5 donor-strain peritoneal cells, either at the time of transplantation, or at 360 days following transplantation

Treatment of CBA Animals	Number of grafts surviving (Time after transplant in Days)	
	0	360
10^5 BALB/c p.c.	0/5	0/6
10^5 CBA p.c.	5/5	ND
No treatment	ND	3/4*

* 1 graft not identified on removal of kidney for technical reasons (see text)

These findings show that cultured BALB/c islet allografts retain transplantation antigen for at least 360 days, and demonstrate that immunisation of the host animal does not take place until these antigens are presented to the host on the surface of viable donor-strain leucocytes. It is also clear that immunological tolerance has failed to develop in animals carrying islet allografts for 360 days, as the host animals have been shown to be capable of mounting an immune response to donor-strain transplantation antigen presented in immunogenic form.

Survival of functional pancreatic islet allografts in streptozotocin-diabetic mice following injection of donor-strain peritoneal cells

Single clusters of cultured mouse pancreatic islets have been shown to carry recognisable transplantation antigen for at least 360 days after allotransplantation, and are readily rejected following challenge with 10^5 donor-strain peritoneal cells. In order to establish whether functional islet allografts can be rejected in similar fashion, attempts were made to induce rejection of pancreatic islet allografts that had maintained sustained reversal of streptozotocin-diabetes.

In a preliminary experiment, 2 CBA mice made diabetic with an intravenous injection of streptozotocin (300mg/kg body weight) were each transplanted with 6 clusters of BALB/c islets (approximately 300 islets) that had been cultured for 7 days beforehand. These allografts largely reversed non-fasting hyperglycaemia within 14 - 21 days of transplantation (Figure 5.1), and at 70 days following grafting, both animals were challenged with an intravenous injection of 10^5 BALB/c (donor syngeneic) peritoneal cells. Within 12 days of this injection, one of the mice had developed marked hyperglycaemia (Figure 5.1), but the second animal showed no sign of rejecting its allograft, as non-fasting blood glucose estimations remained either in or just above the normal range for non-diabetic CBA mice (Figure 5.1). This animal was challenged with a further injection of 10^5 BALB/c

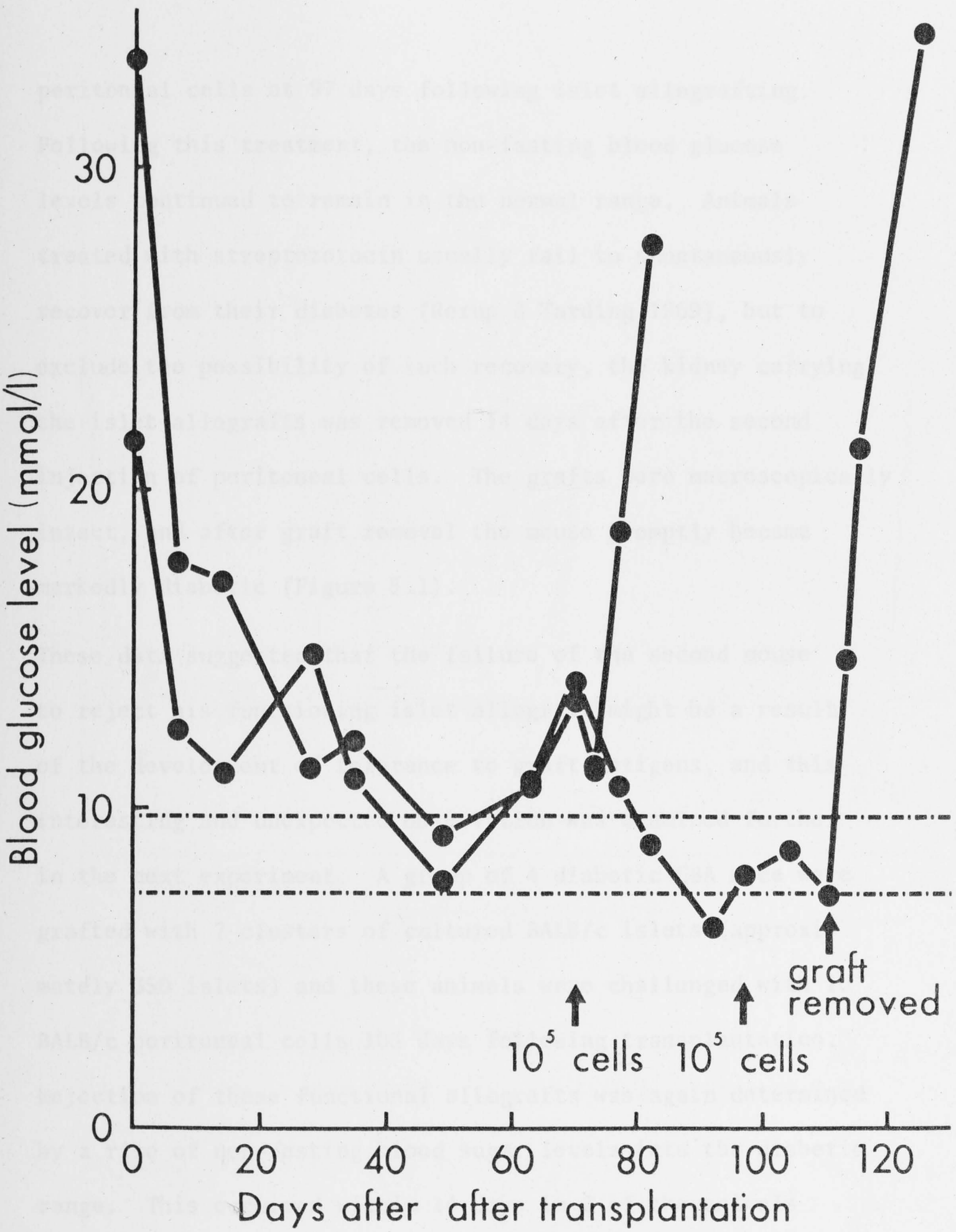


Figure 5.1 Non-fasting blood glucose levels in a group of 2 streptozotocin-diabetic CBA mice returned to near normal levels after transplantation of approximately 300 cultured BALB/c islets. Subsequent challenge with donor-strain peritoneal cells induced graft rejection in one animal. The second animal failed to reject its graft after 2 consecutive challenges with peritoneal cells, but promptly became diabetic after the kidney bearing the grafts was removed. (The dotted line represents the 95% confidence interval for non-fasting blood glucose levels in normal CBA mice)

peritoneal cells at 97 days following islet allografting. Following this treatment, the non-fasting blood glucose levels continued to remain in the normal range. Animals treated with streptozotocin usually fail to spontaneously recover from their diabetes (Rerup & Tarding 1969), but to exclude the possibility of such recovery, the kidney carrying the islet allografts was removed 14 days after the second injection of peritoneal cells. The grafts were macroscopically intact, and after graft removal the mouse promptly became markedly diabetic (Figure 5.1).

These data suggested that the failure of the second mouse to reject his functioning islet allograft might be a result of the development of tolerance to graft antigens, and this interesting and unexpected observation was examined further in the next experiment. A group of 4 diabetic CBA mice were grafted with 7 clusters of cultured BALB/c islets (approximately 350 islets) and these animals were challenged with 10^5 BALB/c peritoneal cells 105 days following transplantation. Rejection of these functional allografts was again determined by a rise of non-fasting blood sugar levels into the diabetic range. This occurred within 14 days in 2 of the animals challenged with 10^5 peritoneal cells (Figure 5.2). However, the other 2 mice remained normoglycaemic despite further challenges with 10^6 and 10^7 peritoneal cells given intravenously at 28 day intervals. These islet allografts were then removed by means of left-sided nephrectomy, and were

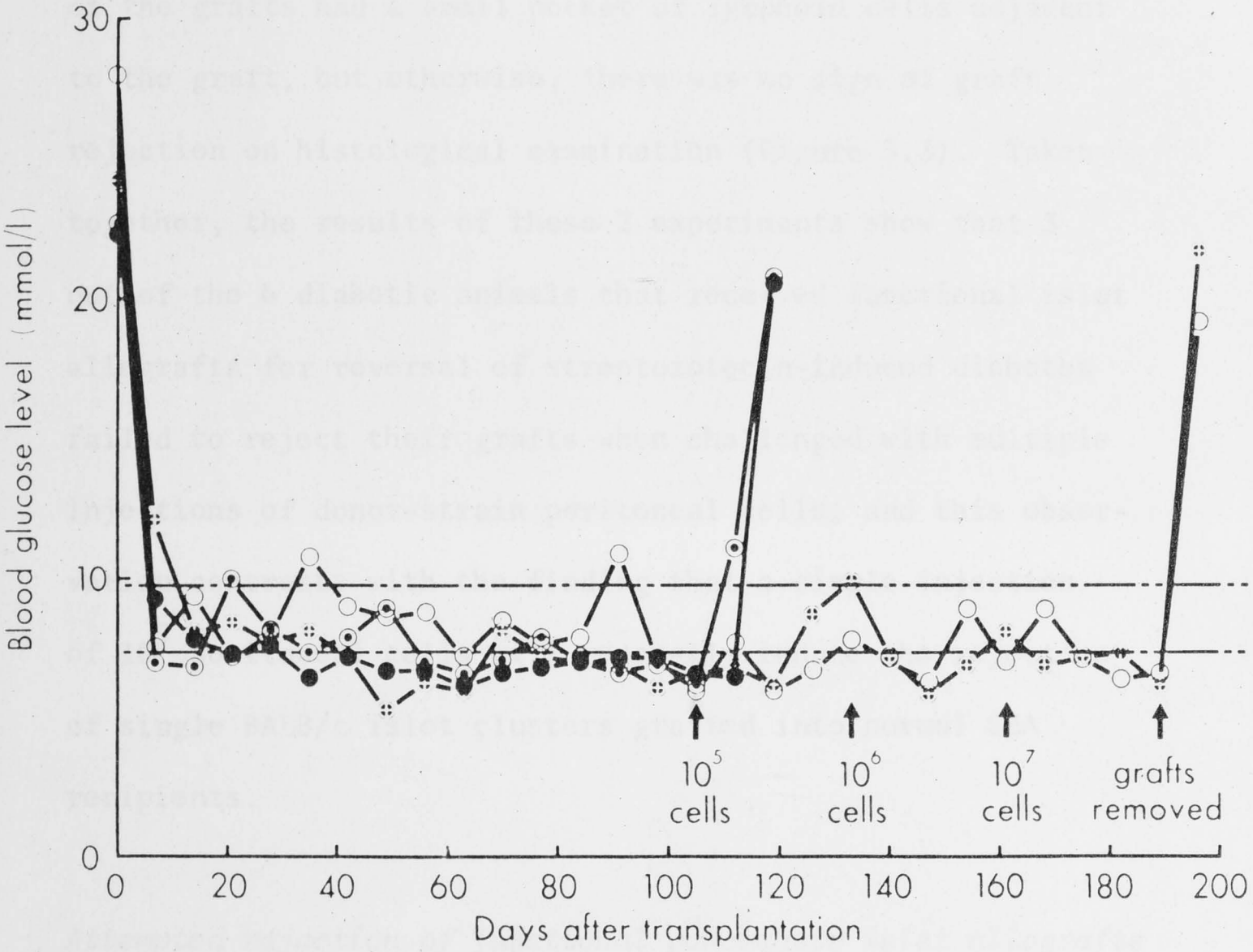
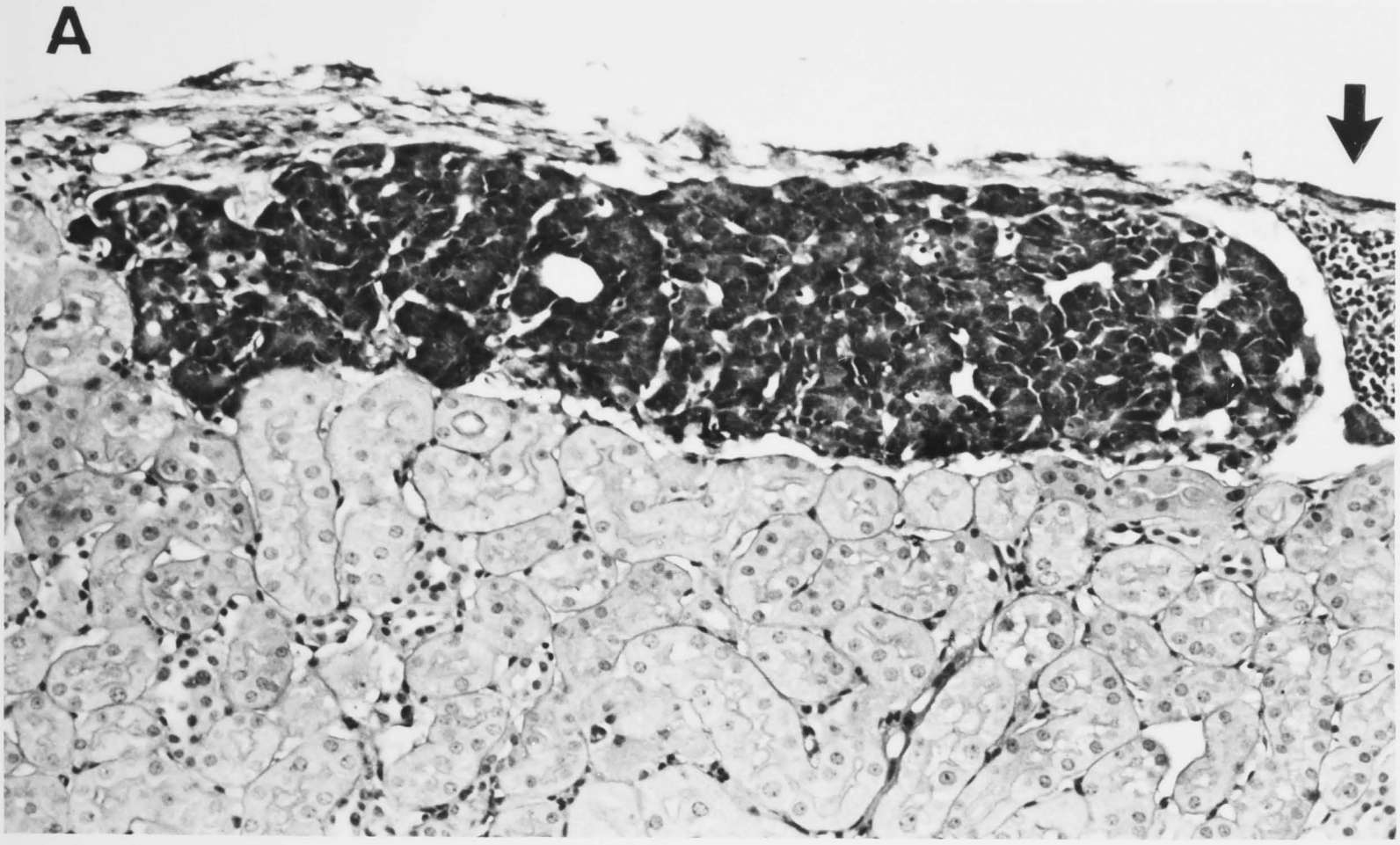


Figure 5.2 CBA animals with functioning long term (105 day) BALB/c islet allografts were challenged with BALB/c peritoneal cells. The grafts were promptly rejected in 2 of the animals when 10^5 cells were injected intravenously, as shown by the return of blood sugar levels to the diabetic pre-transplant levels. The other 2 animals failed to reject their grafts when subsequently challenged with 10^6 and 10^7 peritoneal cells. Surgical removal of these grafts resulted in a prompt return of diabetes. (The dotted line represents the 95% confidence interval for non-fasting blood glucose levels in normal CBA mice)

found to be macroscopically intact. Following graft removal, both animals became diabetic (Figure 5.2). One of the grafts had a small pocket of lymphoid cells adjacent to the graft, but otherwise, there was no sign of graft rejection on histological examination (Figure 5.3). Taken together, the results of these 2 experiments show that 3 out of the 6 diabetic animals that received functional islet allografts for reversal of streptozotocin-induced diabetes failed to reject their grafts when challenged with multiple injections of donor-strain peritoneal cells, and this observation contrasts with the finding that a single injection of 10^5 peritoneal cells will generally induce the rejection of single BALB/c islet clusters grafted into normal CBA recipients.

Attempted rejection of functional pancreatic islet allografts by injection of Freunds complete adjuvant

In 2 separate experiments, a non-specific stimulus to the host immune system was given to a total of 8 CBA mice bearing functional BALB/c islet allografts, in order to see whether non-specific activation of the host immune response would lead to allograft rejection. The first experiment entailed the intraperitoneal injection of 3 CBA mice with 0.2ml of emulsified Freunds complete adjuvant (FCA) at 154 days following allotransplantation of 7 clusters of cultured BALB/c islets. These animals all suffered an abrupt decline in body weight



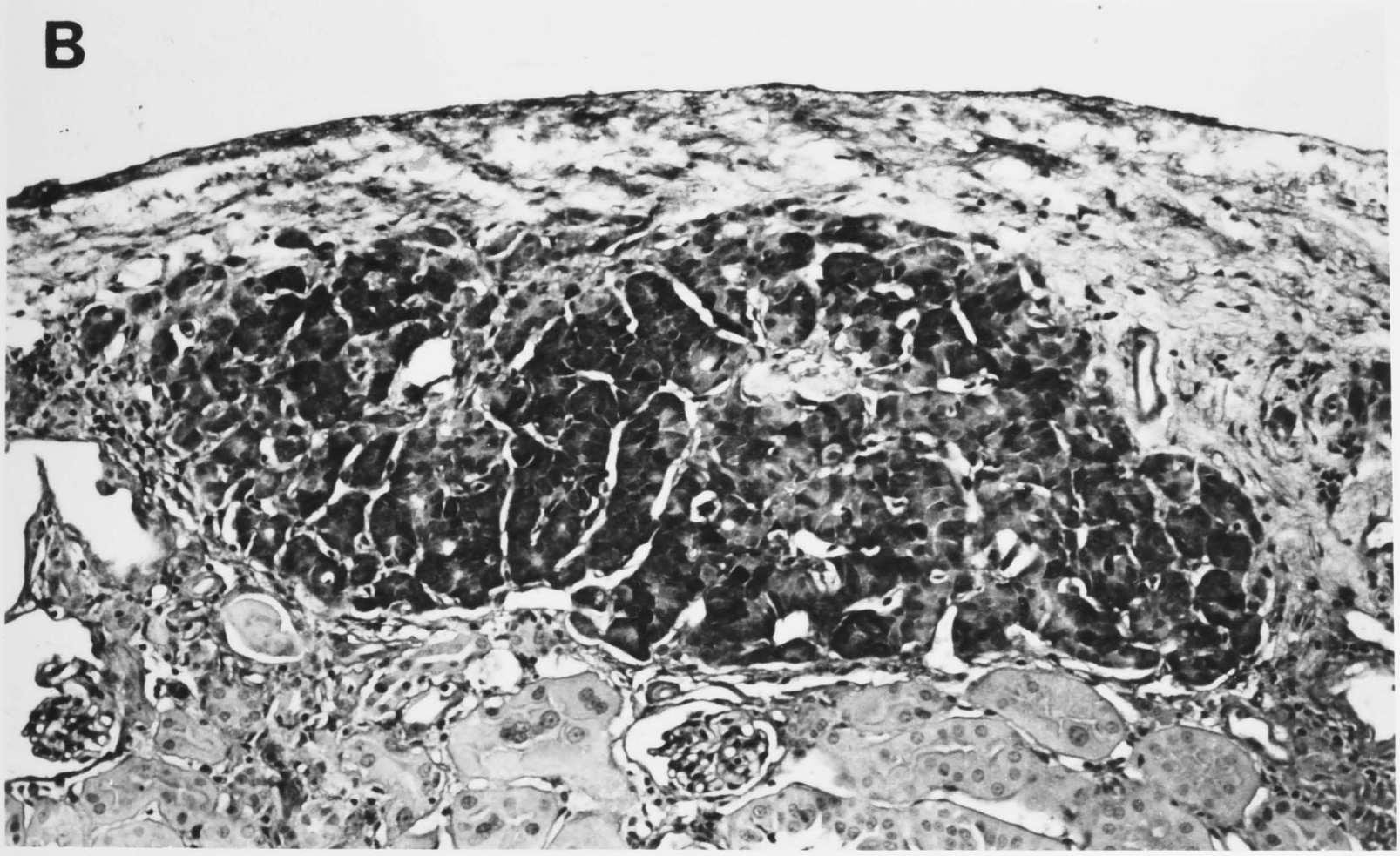


Figure 5.3A and B Representative histological sections of islet allografts removed from the 2 animals that failed to reject their allografts following 3 consecutive challenges with donor strain peritoneal leucocytes. Note staining of beta cells with aldehyde-fuchsin. Apart from a small pocket of lymphocytes seen adjacent to one of the grafts (arrow) there was no sign of an allograft response. (aldehyde-fuchsin x 520)

within a week of injection, but no effect on allograft function was seen. The grafts were removed from the animals 35 days after challenge with FCA. All grafts were macroscopically and histologically intact, and although 1 animal failed to survive the operation, the other 2 promptly became diabetic (Figure 5.4). This experiment was then repeated using a group of 5 CBA mice transplanted with functional BALB/c islets 63 to 112 days beforehand. Again, all animals lost several grams in weight within a week of injection of FCA, but none of the 5 became diabetic. On removal of the grafts 35 days following challenge with FCA, 4 of the animals promptly became diabetic (Figure 5.5). The fifth died at operation. However, the grafts were intact, with no signs of rejection. These results show that an agent known to markedly amplify non-specific immune responses *in vitro* and *in vivo* will not induce rejection of functioning islet allografts in mice made diabetic prior to transplantation by the injection of streptozotocin.

DISCUSSION

Cultured murine thyroid glands lose their immunogenicity after a period in organ culture when exposed to high ambient oxygen tensions, yet retain recognisable transplantation antigen (Lafferty *et al* 1976b; Sollinger *et al* 1977). One of the aims of this present study was to demonstrate whether organ culture produced similar effects when applied to

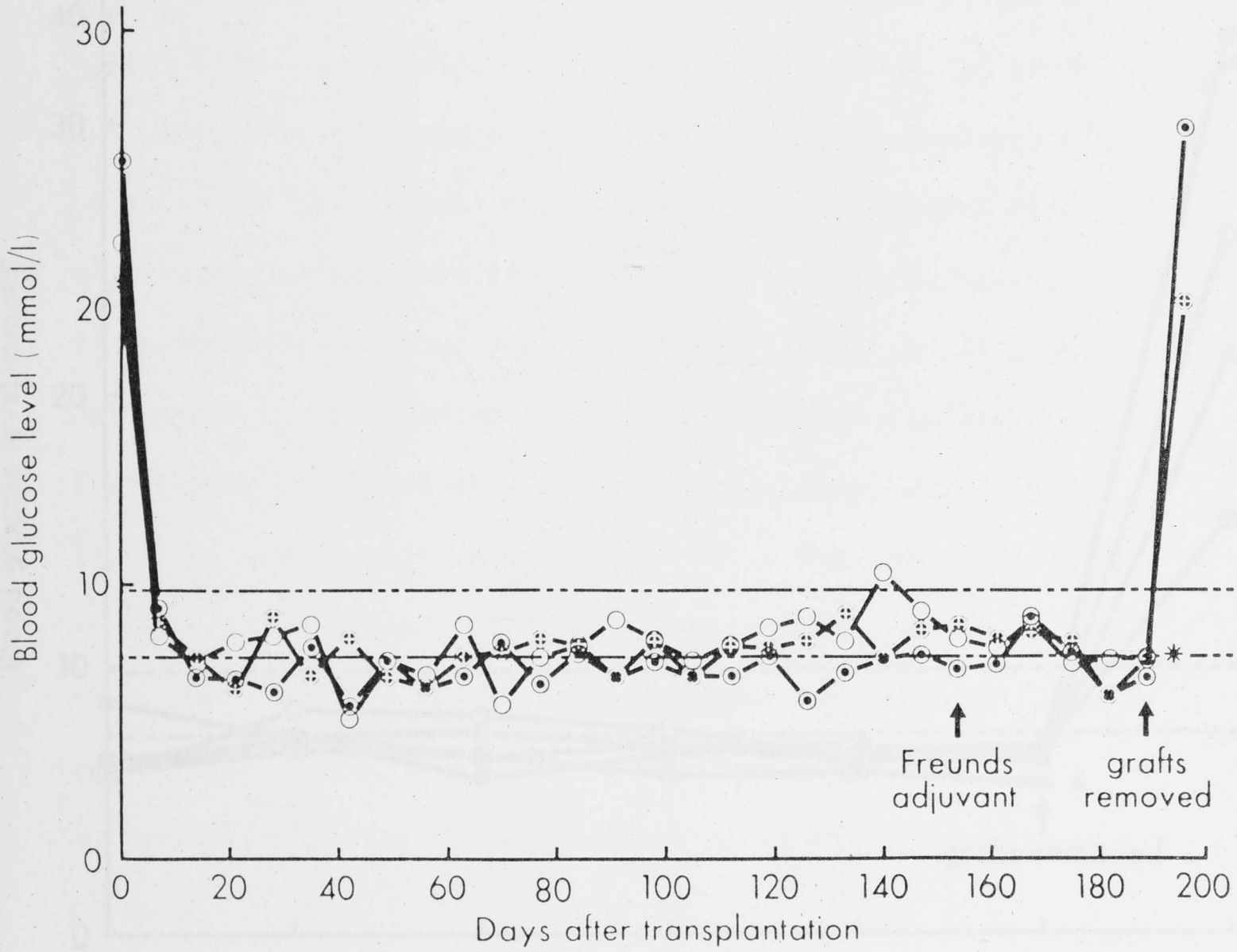


Figure 5.4 Intraperitoneal injection of Freunds complete adjuvant failed to induce rejection of functional BALB/c pancreatic islet allografts in a group of 3 CBA animals challenged 154 days after allotransplantation. Removal of the grafts produced a rapid onset of hyperglycaemia in 2 animals. The third (*) died at operation. The grafts showed no sign of rejection when examined histologically. (The dotted line represents the 95% confidence interval for non-fasting blood glucose levels in normal CBA mice)

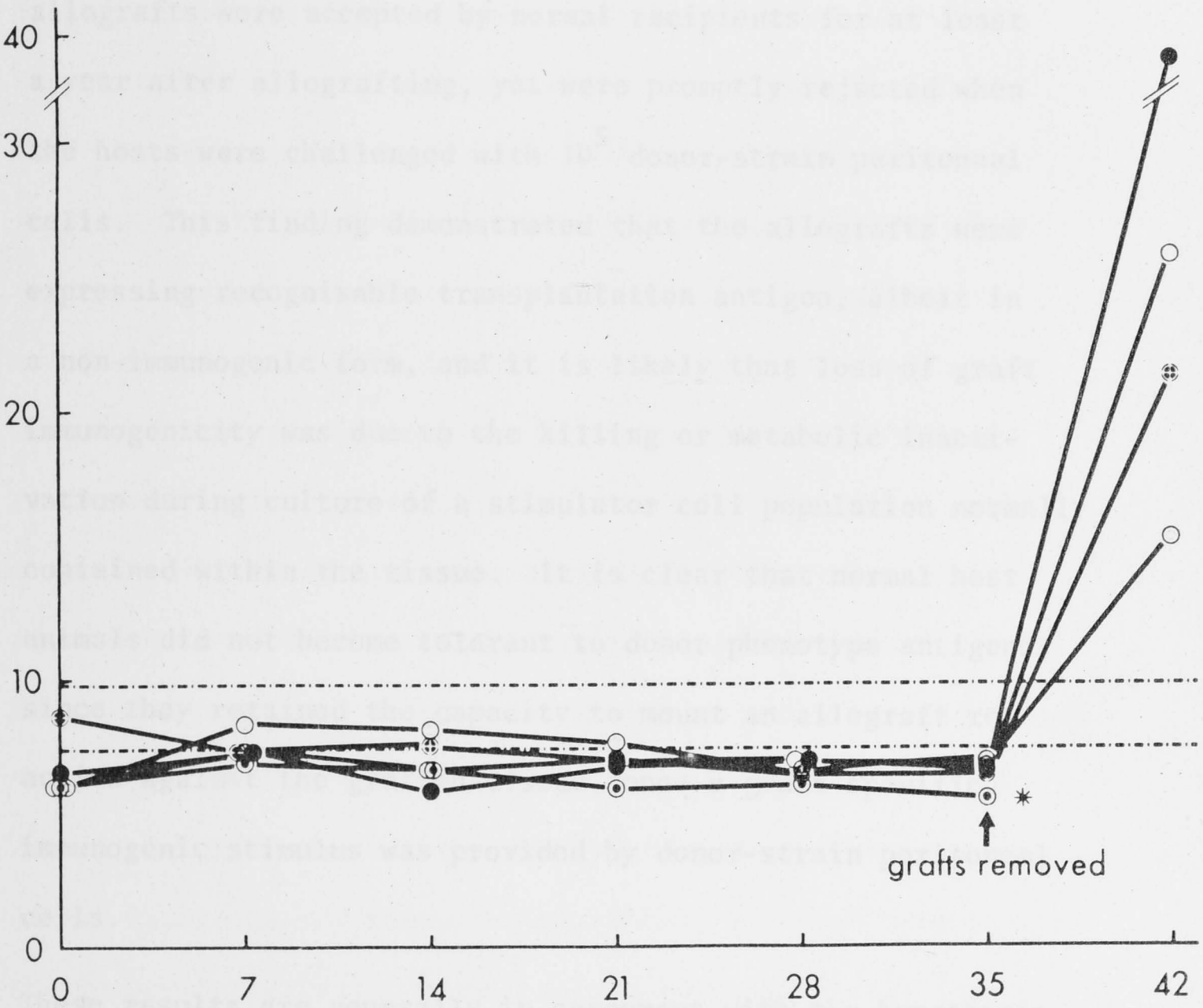


Figure 5.5 Intraperitoneal injection of Freunds complete adjuvant failed to induce rejection of functional islet allografts after a group of 5 CBA animals were challenged 63 to 112 days following transplantation with 0.2 ml of emulsified adjuvant. Removal of the grafts produced a rapid onset of hyperglycaemia in 4 animals. The fifth (*) died at operation. None of the grafts showed signs of rejection. (The dotted line represents the 95% confidence interval for non-fasting blood glucose levels in normal CBA mice)

pancreatic islets prior to allografting. The results show that this is indeed the case, as cultured pancreatic islet allografts were accepted by normal recipients for at least a year after allografting, yet were promptly rejected when the hosts were challenged with 10^5 donor-strain peritoneal cells. This finding demonstrated that the allografts were expressing recognisable transplantation antigen, albeit in a non-immunogenic form, and it is likely that loss of graft immunogenicity was due to the killing or metabolic inactivation during culture of a stimulator cell population normally contained within the tissue. It is clear that normal host animals did not become tolerant to donor phenotype antigens, since they retained the capacity to mount an allograft reaction against the grafted tissue, once a graft-specific immunogenic stimulus was provided by donor-strain peritoneal cells.

These results are generally in agreement with the hypothesis (Lafferty & Cunningham 1975; Bach, Bach & Sondel 1976), that activation of the host response to transplantation antigen depends on a 2-signal mechanism; the first signal being provided by antigen, and the second by a metabolically active stimulator cell of donor-strain phenotype. However, the postulate that chronic exposure to antigen in non-immunogenic form may lead to specific tolerance does not hold in this instance.

As normal CBA mice rejected single clusters of cultured BALB/c

islets following a single challenge with 10^5 donor-strain peritoneal cells, it was anticipated that similar challenge of a group of streptozotocin-treated CBA mice bearing functional BALB/c islet allografts would also result in prompt graft rejection, and a return to the diabetic condition. In 3 out of the 6 animals tested, this proved to be the case, but the other 3 animals failed to reject their grafts after repeated challenge with donor cells. This unexpected result suggested that these 3 animals may have become unresponsive to donor stimulator cells. Lacy, Davie & Finke (1979b) found that a single injection of as many as 10^7 donor-strain peritoneal exudate cells was insufficient to induce rejection of functional rat islet allografts, with 4 out of 6 animals tested requiring 2 or 3 further challenges with up to 6×10^7 peritoneal exudate cells before rejection occurred, but in a more recent study, Lacy's group indicated that 1.5×10^7 donor-strain peritoneal cells were required to consistently induce rejection of functional rat islet allografts by a single cell challenge (Zitron *et al* 1981). Taken together, their results suggest that all 6 mice in the present study may have rejected their functional islet allografts if the initial challenge had been carried out with a larger number of donor-strain peritoneal cells, but despite this possibility, the failure of 3 of these mice to respond to several challenges demonstrates a decreased level of host responsiveness when compared to normal non-diabetic mice allografted with single clusters of cultured islets and subsequently challenged with donor cells.

The disparity in these results might be due to the putative long-term immunosuppressive effects of streptozotocin. The issue is a confused one, with Nichols *et al* (1980) claiming that streptozotocin treatment produces a direct and long-term (greater than 80 day) suppressive effect on the host allograft response, while others maintain that streptozotocin-induced hypoinsulinaemia is responsible for this effect (Friedman & Beyer 1977; Ptak, Rewicka & Kollat 1980; Ishibashi *et al* 1980). Nevertheless, later host unresponsiveness to donor immunogen has been observed in a number of experimental allograft systems when formal immunosuppressive therapy with agents such as antilymphocyte serum or cyclosporin A has been used at the time of allotransplantation (Barker *et al* 1974; Gray & Watkins 1976; Green & Allison 1978; Green, Allison & Precious 1979; White *et al* 1980) and the possibility of streptozotocin inducing similar effects should be taken into account when attempts are made to induce rejection of long surviving islet allografts. Certainly, short-term immunosuppressive effects of the drug have not been obvious, as uncultured islets allografted to streptozotocin-diabetic mice or rats generally reject within days or weeks of transplantation (Reckard & Barker 1973; Gray & Watkins 1976; Weber *et al* 1976; Lacy, Davie & Finke 1979a and b; Chapter 4 of this thesis).

It could also be argued that the amount of islet tissue allografted may also affect the development of tolerance. Calne (1977) has postulated that a large graft of poorly immunogenic

tissue may provide "excess antigen" which may in turn induce tolerance in the host, and Finch & Morris (1977) have provided tentative evidence suggesting that tolerance to large amounts of islet tissue may occasionally occur. In the studies detailed in this Chapter, diabetic animals receiving functional allografts were grafted with approximately 7 times the mass of islet tissue grafted to non-diabetic animals, and the extra quantity of poorly immunogenic transplantation antigen exposed to the host in this situation might lead to the development of tolerance. Although the explanation is purely speculative, the phenomenon needs to be further examined, particularly as allografts of tissues such as ovary (Linder 1962) and liver Houssin *et al* 1980; Kamada, Brons & Davies 1980) have been shown to be capable of inducing specific tolerance in normal naive animals. In practical terms, failure of an allograft reject after the host is challenged with a donor-specific immunogenic stimulus is not a bad thing. If translated to the clinical situation, it could presumably offer protection against initiation of graft rejection if the patient was subsequently exposed to cross-reacting antigens, such as might occur during blood transfusion.

Challenge with Freunds complete adjuvant, which provides a strong non-specific activation of the immune system, failed to induce graft rejection in any of the 8 animals tested, and this result suggests that islet allografts are unlikely

to be rejected as a result of a non-specific activation of the immune system that might occur as the result of infection of the host. However, because of the possible immunosuppressive effects of streptozotocin, such mechanisms may be better studied in the Bio Breeders (BB) rat, (Nakhooda *et al* 1977), a new animal model that develops insulin-dependent diabetes spontaneously.

CHAPTER SIX

CELLULAR MORPHOLOGY, H-2 COMPLEX, AND 1_a ANTIGEN DISTRIBUTION OF MOUSE THYROID GLANDS AND ISLETS OF LANGERHANS : EFFECTS OF ORGAN CULTURE

CHAPTER SIX

INTRODUCTION

It is now evident that successful allotransplantation and indefinite survival of rodent thyroid, parathyroid and pancreatic islet allografts can be achieved if the tissues are conditioned prior to transplantation by a period of organ culture (Lafferty *et al* 1975; Sollinger *et al* 1977; Naji, Silvers & Barker 1979; Lacy, Davie & Finke 1979a and b; Chapter 3 of this thesis). The procedure appears to modulate the immunogenicity of the allograft, although the graft rejection that generally follows after challenge of the host with donor-strain stimulator leucocytes demonstrates that the cultured tissue bears recognisable transplantation antigens. Organ culture appears to modulate graft immunogenicity by having deleterious effects on stimulator cells present within the tissue (Lafferty *et al* 1976b; Lafferty & Woolnough 1977; Chapter 5 of this thesis; Lacy, Davie & Finke 1979b).

Histological studies have indicated that cultured thyroid (Lafferty *et al* 1976b) and islets (Moskalewski 1965; Andersson & Hellerstrom 1972) may also lose their vascular endothelium.

These observations are of particular interest, because vascular endothelium is common to most tissues, and has been reported as possessing stimulator capacity (Hirschberg *et al* 1975), as well as transplantation antigens (Sybesma *et al* 1974; Gibofsky *et al* 1975; Hirschberg, Moen & Thorsby 1979). As such, loss of endothelial cells may in itself lead to a decrease in graft immunogenicity, and might also reduce the susceptibility of the graft to rejection by a sensitised host, since any loss of endothelial cells would mean a corresponding decrease in the amount of transplantation antigen expressed by the graft.

In order to relate cellular changes due to organ culture with the altered immunogenicity of cultured allografts, it is important to know both the stimulator capacity and the degree of expression of H-2 target antigens of the cells involved. Immunoferritin labelling of mouse H-2K/D and Ia antigens has shown expression of these antigens by a variety of defined epithelial cell types (Parr 1979a and b; Kirby & Parr 1979; Parr & Kirby 1979; Parr & McKenzie 1979), and it is possible that the stimulator cells of normal tissues may express Ia antigens, as anti-Ia sera block mixed leucocyte culture reactions (Fish *et al* 1976; Okuda, David & Schreffler 1977). It is reasonable to expect that labelling studies for Ia antigens performed on cells dissociated from normal tissues may provide at least an indirect indication as to which cell types might have the potential to act as stimulator cells. The target antigenicity of the various classes

of tissue cells before and after culture could similarly be determined by labelling the H-2 complex antigens present on the dissociated cells.

The effect of organ culture on cells such as tissue macrophages, fibroblasts, and lymphatic endothelium has not been apparent in previous studies, due to the use of paraffin sections. To gain a clearer picture of the morphological changes that take place during the culture of mouse pancreatic islets and thyroids, a comparative electron-micrographic examination of uncultured and cultured mouse islet and thyroid tissues was carried out, and the immunoferritin labelling technique was applied to defining the distribution of H-2 complex antigens on uncultured and cultured mouse thyroids, and of Ia antigens on uncultured mouse thyroid glands and islets of Langerhans.

The electron-microscopy studies showed that the endothelial cell component of both the thyroid gland and islets of Langerhans was destroyed by organ culture, while the immunoferritin labelling studies demonstrated that the parenchymal and endothelial cells of the thyroid continued to express H-2 complex antigens. Ia antigens were not detected on either thyroid or islet parenchymal cells, but in the uncultured thyroid a minor cell type bearing Ia antigens was found.

RESULTS

Electron microscopy of cultured and uncultured islets of Langerhans

The 4 normal mouse islets of Langerhans studied consisted of endocrine cells and a generous network of blood capillaries lined by fenestrated endothelial cells (Figure 6.1). These findings were consistent with those of an earlier study (Like & Chick 1970). The 7 islets examined that were obtained from animals treated with cyclophosphamide were indistinguishable from normal islets. After 7 days in culture, capillary endothelial cells could not be distinguished in any of the 9 islets examined (Figure 6.2). Occasional pyknotic nuclei from degenerating cells were present, and these probably represented the last traces of endothelium. Small intracellular spaces between endocrine cells probably represented the former capillary lumina, and contained cellular debris (Figure 6.3). Electron microscopy showed no abnormality of the endocrine cells. Islet clusters cultured for 14 days *in vitro* were also devoid of vascular endothelium, and most endocrine cells appeared viable.

Electron microscopy of cultured and uncultured thyroid glands

The 11 normal thyroid lobes studied consisted mainly of follicles that were surrounded by a dense network of blood vessels. Adipose cells, lymphatics and the occasional fibroblast were present in the interfollicular connective tissue

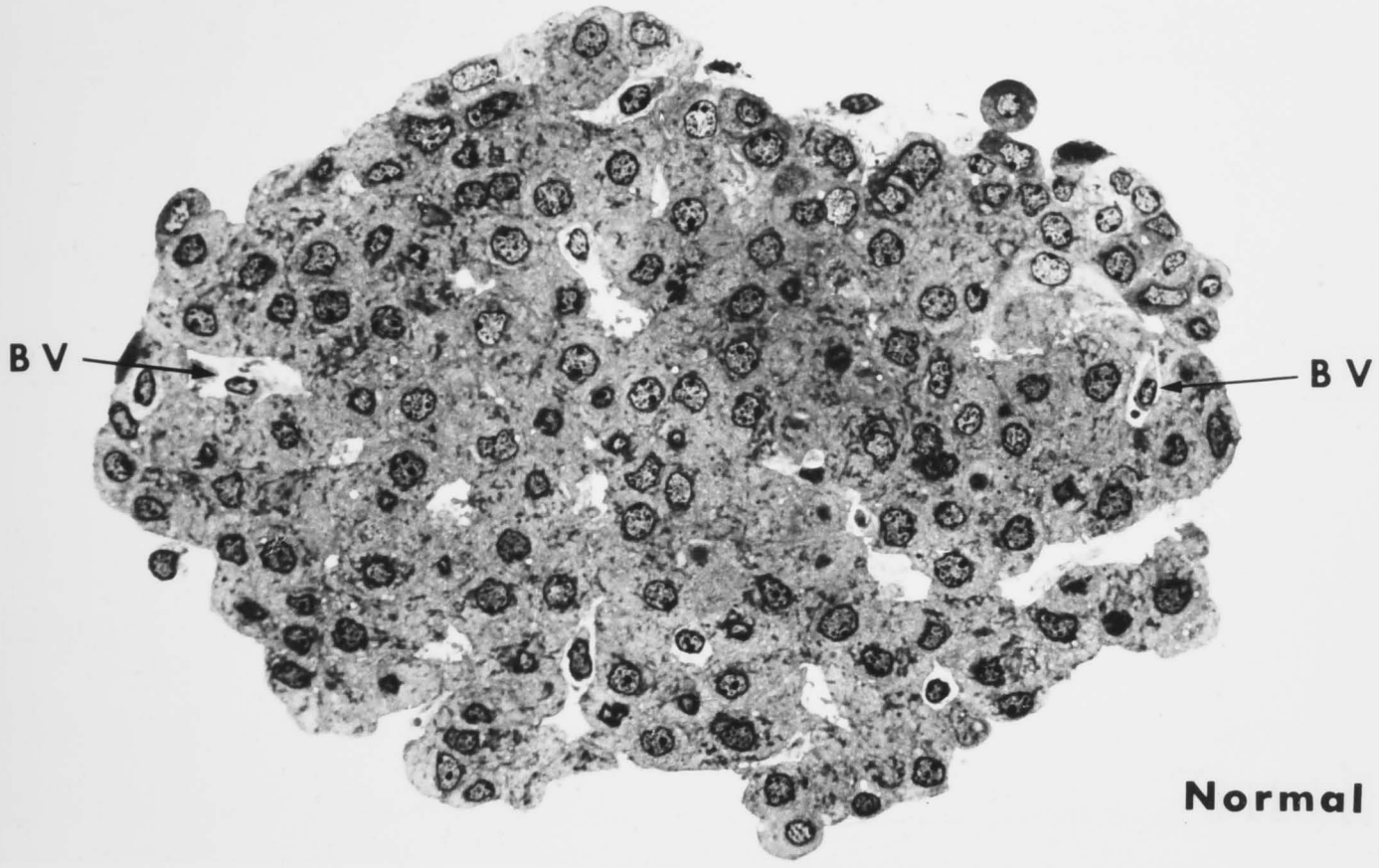


Figure 6.1 Normal mouse islets of Langerhans dissociated from the pancreas by collagenase digestion, showing numerous capillary endothelial cells (BV) distributed among the endocrine epithelial cells (x 500)

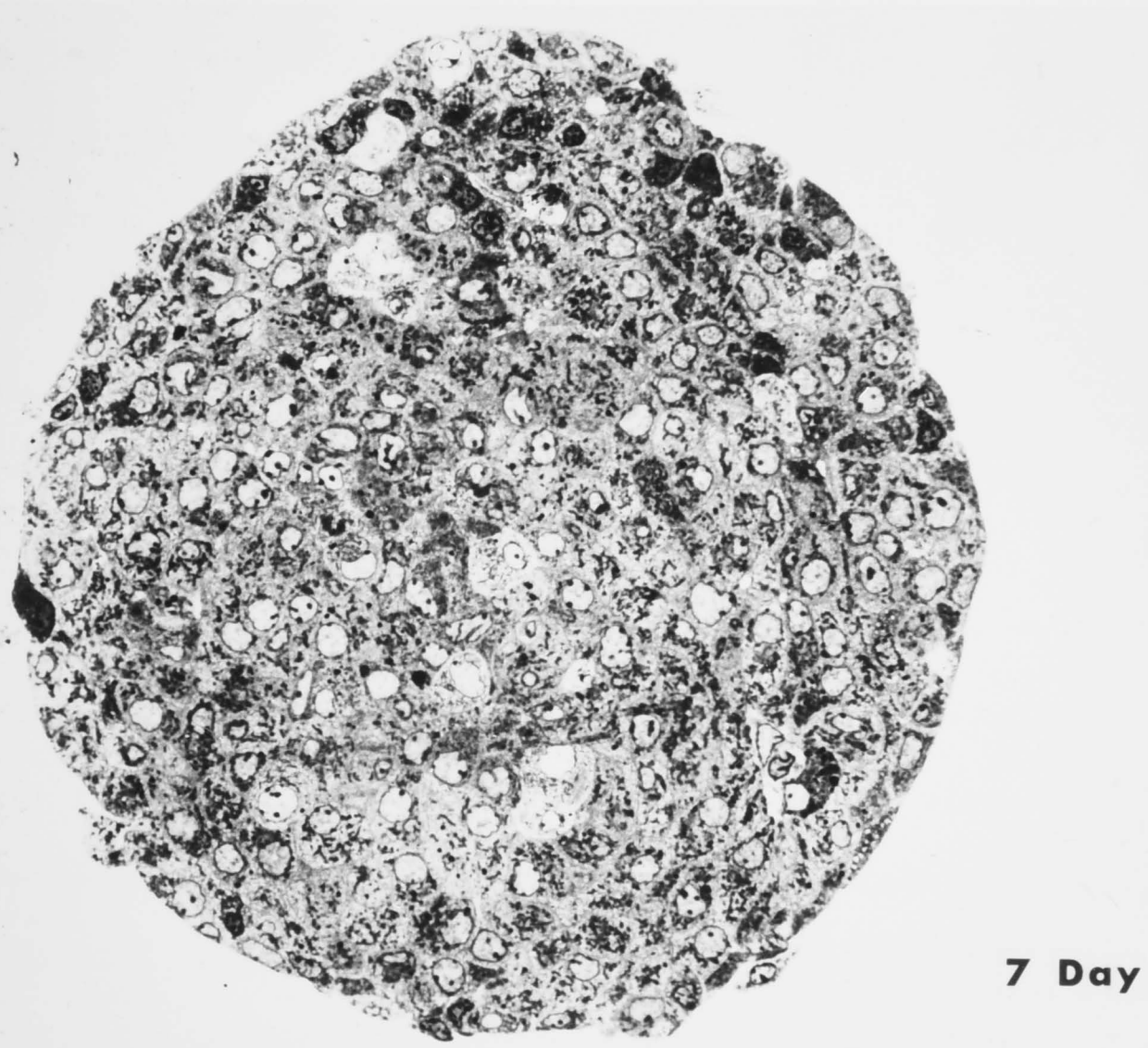


Figure 6.2 Islets of Langerhans after 7 days of culture. Light and electron microscopy showed all cells to be endocrine in type, with no viable or degenerating capillary endothelial cells seen (x 500)

space (Figure 6.4) and these findings were consistent with those previously described by Ekholm (1964). The 9 thyroid lobes from animals pre-treated with cyclophosphamide were indistinguishable from normal thyroids.

After 7 days in culture, thyroid follicles in all 9 lobes studied remained normal, but there was extensive deterioration of cells in the inter-follicular space (Figure 6.5). However, some dead endothelial cells were still recognisable, and others appeared to be almost normal (Figure 6.6). Most adipose cells were dead, but cells that appeared to be fibroblasts remained viable; these may have been even slightly enlarged. They contained abundant rough endoplastic reticulum, and an accumulation of large, dense granules. The follicles remained well separated and at low magnification the partial deterioration of the inter-follicular tissue gave the impression of empty spaces between the follicles (Figure 6.5). After 14 days in culture the lymphatics and adipose cells in the 8 lobes studied had disappeared, while a few poorly preserved vascular endothelial cells still remained within the inter-follicular spaces (Figure 6.7). Viable cells containing substantial amounts of rough endoplastic reticulum and probably lysosomal granules were seen within the inter-follicular spaces, and these were probably fibroblasts. Because of the loss of inter-follicular tissue, the follicles appeared tightly packed, especially at the periphery of the glands. Follicle epithelial cells appeared

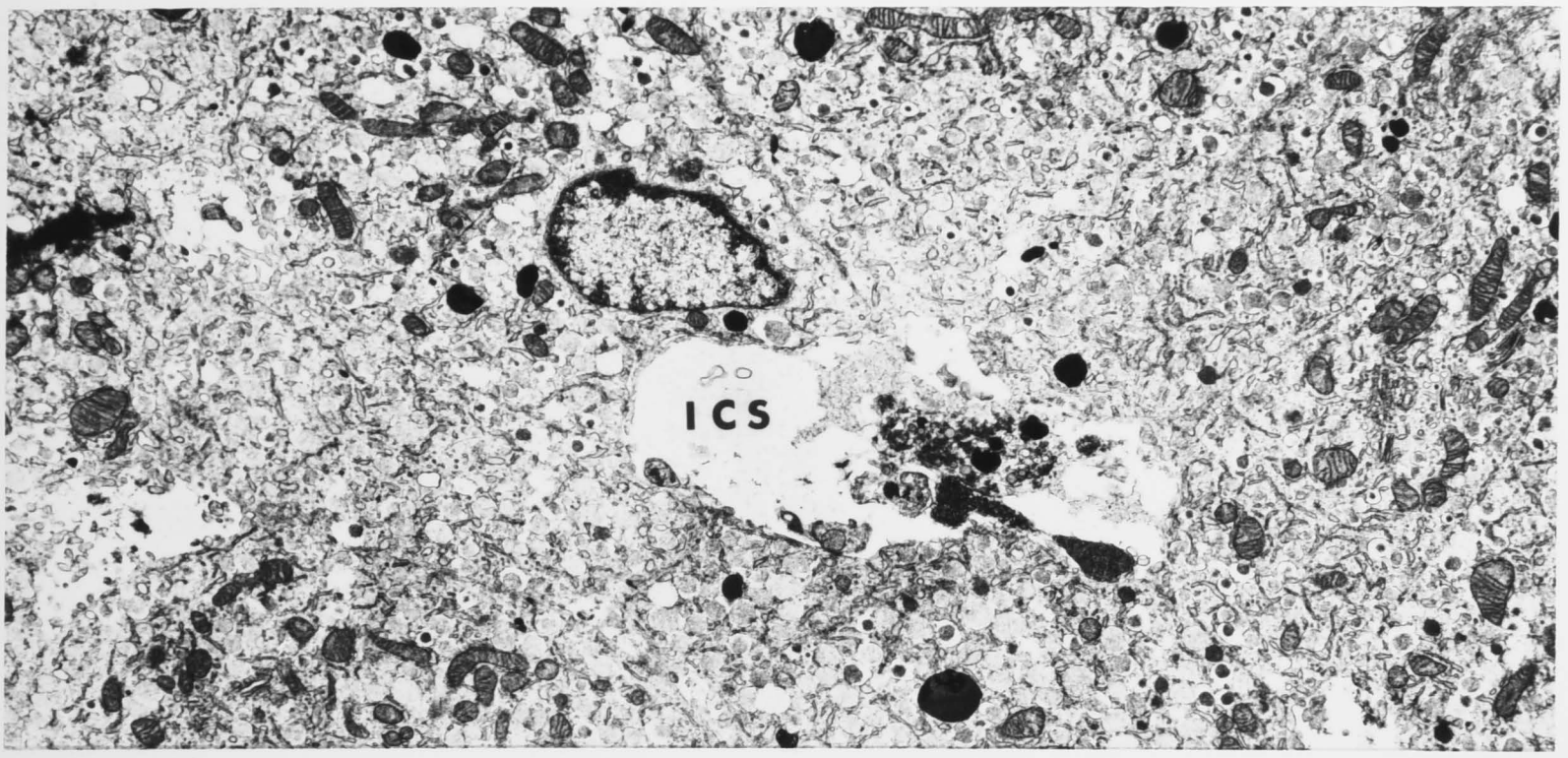


Figure 6.3 A small opening in the intra-cellular space of an islet of Langerhans cultured for 7 days. The cellular debris probably resulted from the degeneration of capillary endothelial cells. No intact endothelial cells were found (x 6,400)

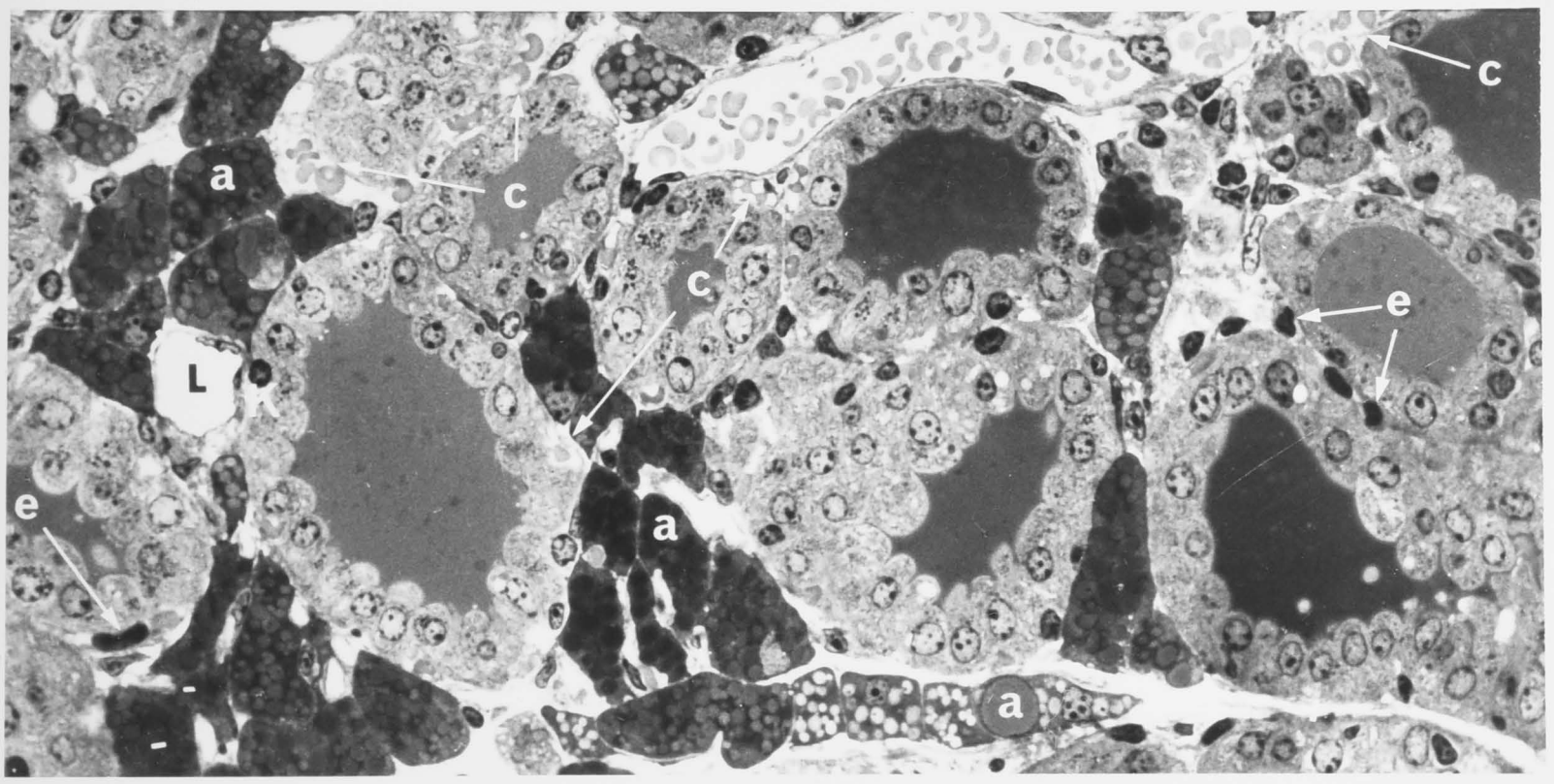


Figure 6.4 Normal histology of the mouse thyroid gland. A network of capillaries surrounds each follicle (c). The nuclei of the endothelial cells (e) lining these capillaries are small and more darkly stained than the nuclei of follicle epithelial cells. Large numbers of adipose cells (a) are scattered throughout the gland. These cells are extremely large and are somewhat unusual in that their lipid is distributed as numerous small droplets rather than one large droplet. The adipose cells are not readily recognised in paraffin sections because their lipid is lost. Lymphatic vessels (L) were common (x 500)

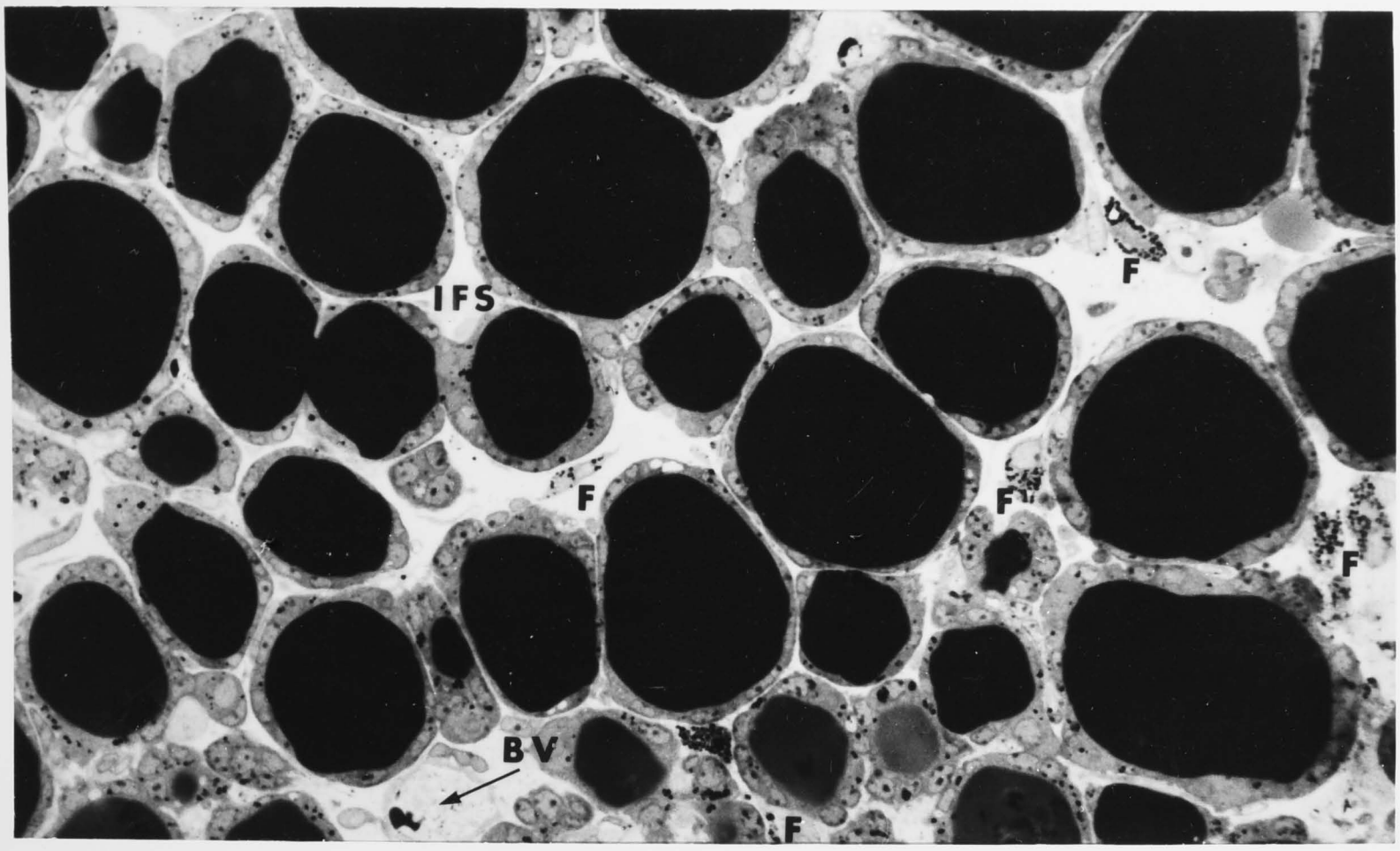


Figure 6.5 Thyroid glands cultured for 7 days contained enlarged follicles, with flattening of the lining epithelial cells. Interfollicular spaces (IFS) appeared empty, due to a loss of blood vessels, lymphatics and adipose cells. Degenerating blood vessels (BV) were present. Fibroblasts (F) in the interfollicular space showed an accumulation of dense granules and a probable increase in cell size (x 500)

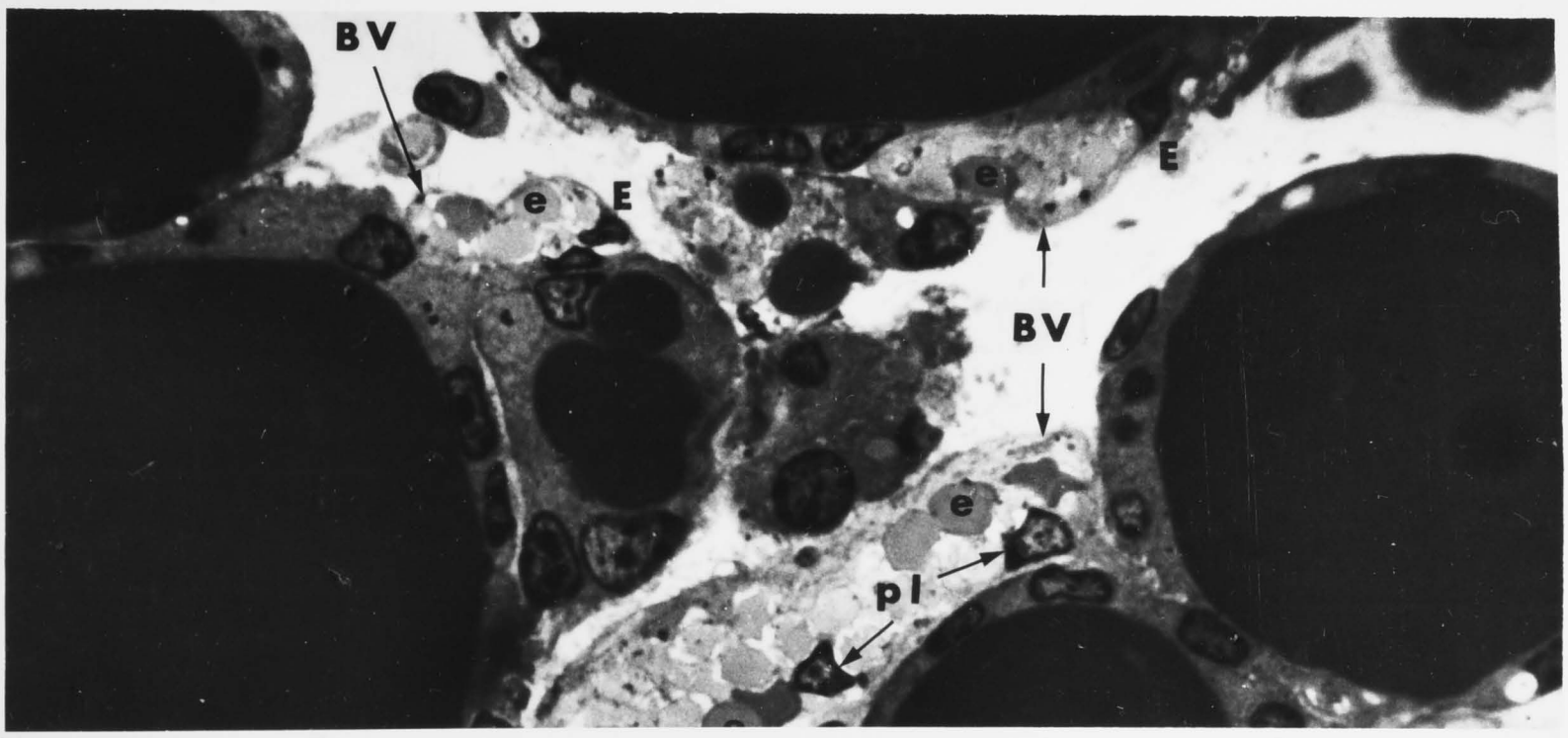


Figure 6.6 A thyroid cultured for 7 days, showing blood vessels (BV) in varying stages of degeneration. Several recognisable capillaries and a small venule are present. Erythrocytes (e) are in various stages of lysis, as indicated by the variable staining density of their cytoplasm, and several endothelial cells (E) can be seen. Two cells in the venule are probably leucocytes (pl); they have more cytoplasm than typical endothelial cells (x 1250)

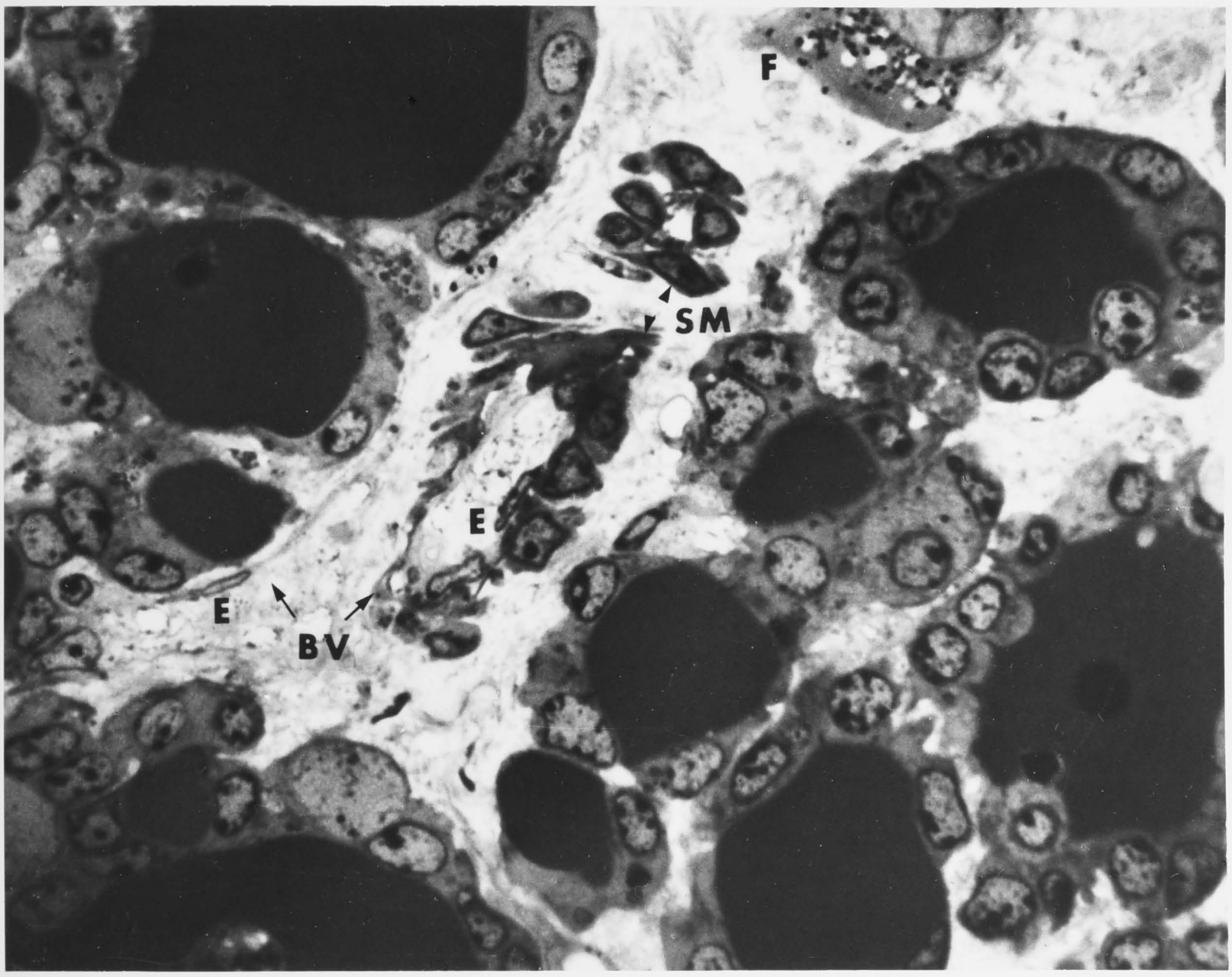


Figure 6.7 A 14 day cultured thyroid gland, showing degenerated blood vessels (BV), fewer in number when compared with 7 day cultured thyroid. Several endothelial cells (E) can still be recognised, but the erythrocytes in the vessel lumina have degenerated. The smooth muscle cells (SM) of the arteriole are well preserved, and a large fibroblast is present at top right (x 1250)

viable, but showed no endocytic activity at the apical membrane, and there were few colloid droplets in the cytoplasm. These cells also showed an accumulation of dense lysosome-like granules. Parathyroid tissue was often seen adjacent to the thyroids, and it too showed a loss of vascular endothelial cells following 14 days of culture.

The distribution of H-2 complex antigens on cultured and uncultured mouse thyroid glands

Collagenase digestion of minced thyroid glands yielded a preparation of dissociated thyroid follicles, and short segments of blood vessels. Additional dissociation of the collagenase digest with trypsin and EDTA after fixation in PLP yielded mainly follicle epithelial cells and capillary endothelial cells, either singly or in small aggregates. Also present were small numbers of other cell types. The endothelial cells retained their characteristic shape, and encompassed capillary lumina, which occasionally contained lysed erythrocytes. Follicle epithelial cells also retained their characteristic shape, which included the segregation of their surface membranes into distinct apical and latero-basal regions. The labelling of H-2 complex antigens on dissociated C57B1/10Sn thyroid cells showed a moderate to dense expression of these antigens on the luminal and ab-luminal surfaces of capillary endothelial cells (Figure 6.8). Follicle epithelial cells failed to take up label on their

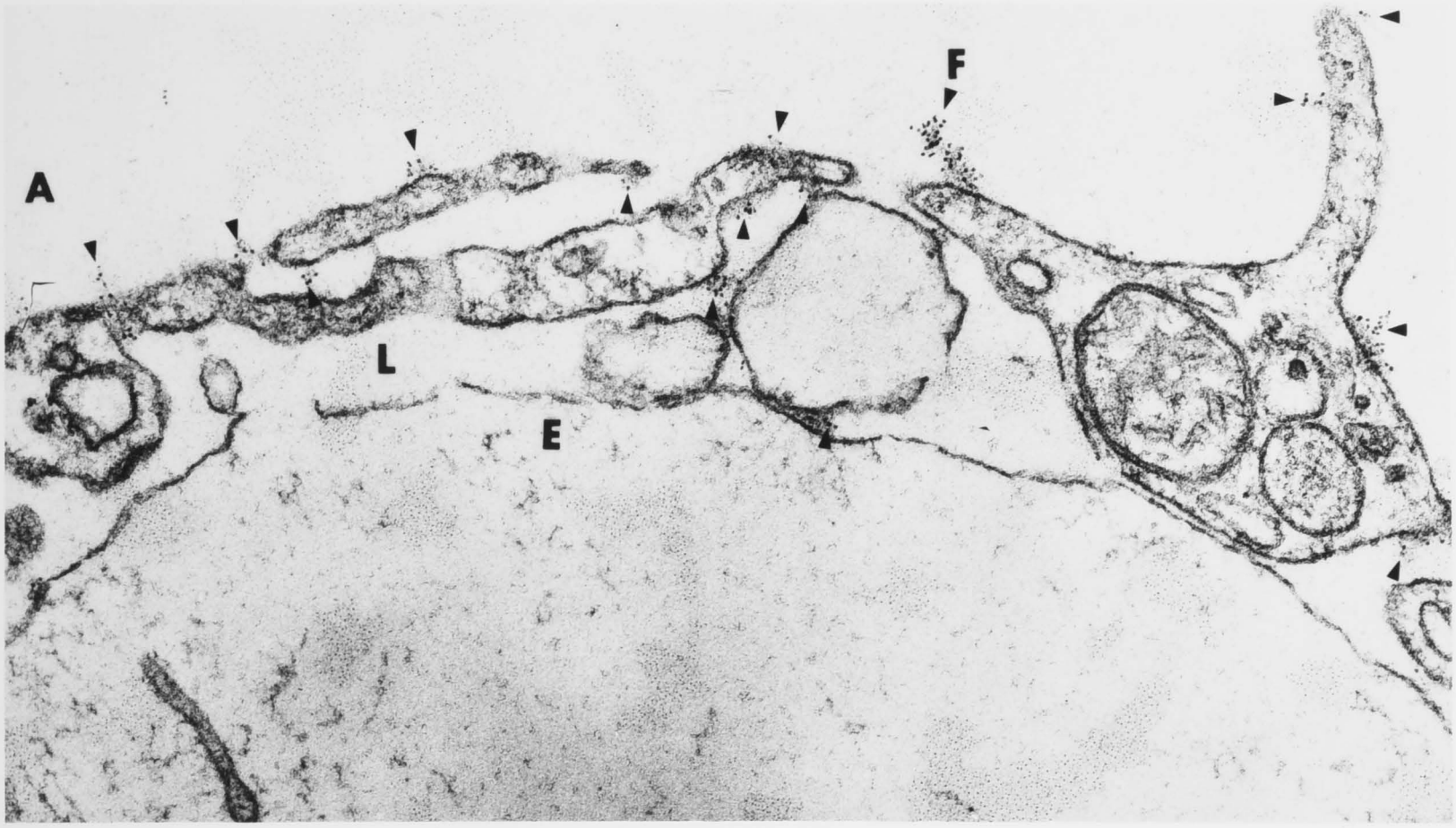


Figure 6.8 Moderate to dense ferritin (F) labelling of H-2^{b} antigens on the fenestrated capillary endothelial cells from normal C57BL/10Sn mouse thyroid glands. Both the luminal (L) and abluminal (A) aspects of the surface membrane are labelled. The membrane of a lysed erythrocyte (E) is present in the lumen (x 64,000)

apical microvillus membranes, but were very lightly labelled on their lateral and basal membranes. Control cell preparations from B10.BR/SgSn showed negligible ferritin binding. Other thyroid cell types, such as fibroblasts and lymphatic endothelium were not identified with certainty in the dissociated cell preparations. Thyroid cells from mice pretreated with cyclophosphamide showed the same labelling results as those from normal mice. Dissociation of 14 day cultured thyroid glands yielded follicle epithelial cells, but no detectable endothelial cells. The density of H-2 complex antigens on the cultured follicle epithelial cells was indistinguishable from that on cells from uncultured glands (Figure 6.9) but no cells bearing dense ferritin labelling were seen, which suggested that both endothelial cells and lymphoreticular cells (Parr 1979a) had been destroyed by the culturing procedure.

The distribution of Ia antigens on uncultured thyroid glands and islets of Langerhans

Labelling of Ia^k antigens on cells dissociated from uncultured B10.BR/SgSn thyroids revealed that neither the follicle epithelial cells nor the capillary endothelial cells express detectable Ia antigens (Figure 6.10). However, a minor cell type that was present exhibited heavy labelling of Ia antigen (Figures 6.10 and 6.11). The labelled cells exhibited a deeply indented nucleus with peripheral hetero-

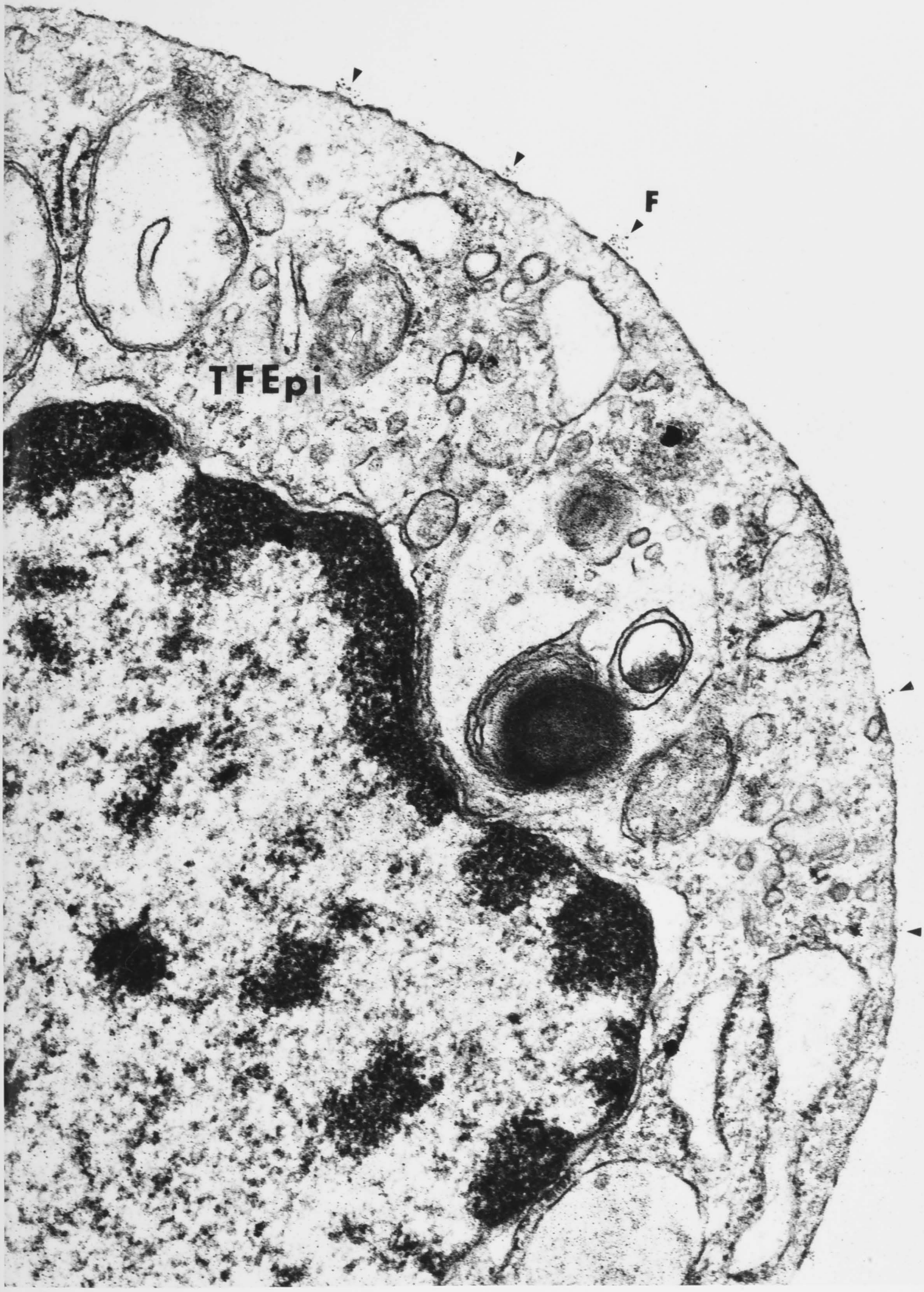


Figure 6.9 A follicle epithelial cell (TFEpi) obtained from a C57BL/6 thyroid gland cultured for 14 days shows ferritin labelled H-2b antigens (F) on its lateral membrane. The labelling density on cultured follicle epithelial cells was indistinguishable from that of normal cells (x 64,000)

chromatin and small nucleolus, abundant cytoplasm containing many small vesicles, sparse rough endoplasmic reticulum, and no lipid droplets or dense residual body lysosomes. Multivesicular bodies, scattered mitochondria, and distinctive cell-surface projections were seen, and the cells showed coated vesicles and invaginations of the surface membrane, which indicated endocytic activity. These uniform findings indicated that the cells labelling with antiserum specific for Ia antigens were of a single type, which is unlikely to be a resident tissue macrophage because of the absence of lipid droplets and residual body lysosomes. These cells did not resemble thyroid fibroblasts, as they had little rough endoplastic reticulum. Endocrine cells and capillary endothelium cells dissociated from B10.BR/SgSn islets of Langerhans showed no labelling of Ia^k antigens. Table 6.1 summarises the overall results.

TABLE 6.1 Expression of H-2 complex and Ia antigens on cells of thyroid glands and islets of Langerhans

Cell Type	H-2 complex	Ia
Thyroid follicle epithelium	(weak) +	-
Thyroid capillary endothelium	+	-
Thyroid minor cell type	?	+
Islet beta cells	- (a)	- (b)
Islet capillary endothelium	+ (a)	-

(a) Parr 1979

(b) Parr & McKenzie 1979, and the present study

DISCUSSION

This study has clarified the situation regarding most of the cellular changes that take place in the thyroid gland and pancreatic islets of the mouse during culture *in vitro*. The vascular endothelial cells and blood cells within the vascular lumina of the thyroid degenerated after 14 days in culture, whereas islets of Langerhans lost their capillary endothelium and any passenger leucocytes remaining after islet isolation by 7 days. Since most of the mobile blood cells apparently were washed out of the islet capillary lumina during the isolation procedure, it can only be assumed that any such cells remaining underwent degeneration during the culture period. Thyroid adipose cells and lymphatic endothelium also degenerated during culture, whereas the endocrine cells of thyroid and islets remained intact. Thyroid fibroblasts also appeared to remain viable. The retention of endocrine epithelial cells and loss of vascular endothelium reported in previous studies (Moskalewski 1965; Andersson & Hellerstrom 1972; Lafferty *et al* 1976b) are thus confirmed by the present observations. Previous studies with cultured thyroid allografts and xenografts indicated that stimulator cells are lost during culture (Lafferty *et al* 1976b; Sollinger *et al* 1977) and data presented in Chapter 5 of this thesis, together with that of Lacy, Davie & Finke (1979b) suggests that these cells are lost during culture of pancreatic islets. The present study shows that fibroblasts and follicle epithelium

are unlikely to act as stimulator cells, as they were not lost from the thyroid during culture, while apidocytes are unlikely to be significant as stimulators because they are rarely seen in tissues such as the islets of Langerhans. On the basis of the histological observations, possible stimulator cell types are passenger leucocytes, vascular endothelium, and lymphatic endothelium. Lymphocyte preparations from various sources have been shown to lose their ability to stimulate if held in culture media for several days prior to being placed in mixed lymphocyte culture (Opelz & Terasaki 1974; Miller, Lifton & Hattler 1975; Davidson 1977), and these findings are consistent with the notion that passenger lymphoid cells carried by an allograft provide a major immunogenic stimulus to graft rejection, and that these cells are lost during organ culture *in vitro*.

Endothelial cells from uncultured mouse thyroid glands showed medium to heavy labelling of H-2 complex antigens that was similar in density to that seen with peritoneal cells and some spleen cells (Parr 1979a). However, thyroid follicle epithelium showed a much lower labelling density. Parr (1979b) has previously shown that the endothelium from islets of Langerhans shows dense expression of H-2 complex antigens. However, he was unable to detect these antigens on isolated beta cells. Since vascular endothelium is a substantial component of uncultured islet and thyroid tissues, its loss during culture may also be associated with reduction

of allograft immunogenicity. Hirschberg, Moen & Thorsby (1979) have reported that human umbilical cord cells express Ia-like antigens and have also claimed (Hirschberg *et al* 1975) that vascular endothelial cells have stimulator activity, as they found that vascular endothelial cells cultured for up to 9 weeks retain the capacity to stimulate allogeneic lymphocytes. They thereby considered that their endothelial cell preparations were free of contaminating stimulator leucocytes, since leucocyte preparations in culture have been shown to lose their stimulatory capacity over a much shorter period of time (Opelz & Terasaki 1974; Miller, Lifton & Hattler 1975; Davidson 1977). On the other hand, data presented in this Chapter shows that cyclophosphamide pretreatment of donor animals had no discernable effect on thyroid or islet vascular endothelium, and a previous study showed that 30% of uncultured thyroid allografts taken from donors pretreated with cyclophosphamide failed to reject when transplanted to naive recipients (Lafferty *et al* 1976a). This result suggested that the most significant stimulator cells in thyroid allografts are found within the population of passenger leucocytes. Although the present study shows that vascular endothelial cells fail to express detectable amounts of Ia antigen, this finding does not necessarily preclude endothelial cells from having a stimulator capacity, for there are at least two mouse tumour cell lines that carry no identifiable Ia antigen, and yet have the capacity to stimulate *in vitro*

(Frelinger *et al* 1974; Batchelor, Welsh & Burgos 1978; Lafferty *et al* 1978). Nevertheless, if endothelial cells do have any stimulator activity, it is likely to be of secondary importance to that of passenger stimulator leucocytes. Despite this, the loss of endothelial cells during organ culture may protect the graft against hyper-acute rejection by a sensitised host (Kissmeyer-Nielsen *et al* 1966; Batchelor *et al* 1979).

Attempts to identify possible stimulator type cells by means of Ia antigen labelling showed that neither endocrine epithelial cells nor endothelial cells expressed detectable Ia antigen, and this finding held for both the thyroid and islets of Langerhans. However, a minor cell type within the thyroid exhibited dense Ia labelling. The ferritin-antibody conjugate used in this labelling study binds to the membrane immunoglobulin of B lymphocytes, but the labelled cell seen in the thyroid preparations was quite different from the B lymphocytes previously observed in the spleen (Parr & McKenzie 1979). The splenic B lymphocytes identified by labelled surface immunoglobulin had a smaller volume of cytoplasm, a more uniformly oval nucleus, and fewer small vesicles. They also lacked multi-vesicular bodies and surface projections. The ultra structural characteristics of the minor cell type are similar to those of splenic dendritic cells (Steinman & Cohn 1973) which are known to express Ia antigen (Steinman, Kaplan & Witmer 1979). The attempted labelling of cell preparations from islets of

Langerhans with anti-Ia^k serum and conjugate revealed only one labelled cell, which appeared to be a B lymphocyte. This cell, and the minor cell type found in thyroid preparations were probably passenger leucocytes.

The immunoferritin labelling technique described here provides a useful means of determining the distribution of transplantation antigens, but experimental data with *in vivo* systems suggest that the sensitivity of the method precludes detection of small but biologically significant quantities of transplantation antigen. Gose & Bach (1979) have shown that Ia antigens are expressed on a thyroid parenchymal cell type that does not have significant stimulator activity, by demonstrating that cultured thyroid glands allografted to recipients differing only at the I-A subregion of the H-2 complex are rejected only after challenge of the recipient with donor-syngeneic lymphoid cells, and although the immunoferritin labelling technique fails to detect H-2 antigens on islet cells (Parr 1979b), Faustman *et al* (1980) have used a complement-dependent cytotoxicity assay to demonstrate that dispersed mouse pancreatic islet cells carry recognisable quantities of H-2K and H-2D antigens, but no Ia determinants. These results show that the techniques used to qualitate and quantitate the presence of transplantation antigens on tissues are really complimentary to each other.

In summary, the experimental results detailed in this Chapter demonstrate that organ culture of mouse thyroid and islets

in a 95% oxygen gas phase leads to selective destruction of the endothelial components of the organs. In addition, they provide suggestive but inconclusive evidence that lympho-reticular cells of stimulator phenotype are destroyed by this form of organ culture and show that parenchymal cells of the mouse thyroid gland continue to express H-2 complex antigen after organ culture.

CHAPTER SEVEN

THE EFFECTS OF ORGAN CULTURE ON THE IMMUNOGENICITY OF MOUSE PANCREATIC ISLETS AND FOETAL PANCREAS : A COMPARATIVE STUDY

CHAPTER SEVEN

INTRODUCTION

It is now clear that organ culture in a gas phase of 95% O_2 will reduce the immunogenicity of adult mouse pancreatic islets (Chapter 3) enabling the reversal of experimental diabetes (Chapter 4), and that host responsiveness to transplantation antigens carried on these allografts can be induced by the intravenous injection of donor-strain peritoneal cells at the time of transplantation (Chapter 5). Peritoneal cell preparations consist mainly of macrophages and lymphocytes (Gesner & Howard 1967) and these cells have the capacity to stimulate cellular immune responses (Greineder & Rosenthal 1975; Talmage *et al* 1976). Thus, it appears likely that organ culture destroys or inactivates those classes of donor cells which have the capacity to stimulate the host immune response to adult mouse pancreatic islet allografts.

Isolated pancreatic islets are fairly readily obtained from adult mice using the technique of collagenase digestion, but the yield is low. The 8 week old mouse pancreas has 5 - 600 islets (Bunnag 1966) but 8 - 10 donor animals are necessary to provide the 350 islets required for complete

reversal of experimental diabetes in the mouse (Chapter 4). The successful use of pancreatic islet allografts to reverse experimental diabetes is encouraging, but the use of isolated pancreatic islets in clinical transplantation studies is severely hampered by the problem of low islet yield. Islets are not readily separated from the human pancreas, as it is more fibrous than its rodent counterpart, and the use of isolated human pancreatic islets for clinical purposes will not become practical until isolation procedures improve (Scharp *et al* 1980).

An alternative approach is to use foetal pancreas. In rodents, foetal pancreas provides an excellent source of endocrine tissue, and isografts have been shown to be capable of reversing experimental diabetes (Brown *et al* 1976). When compared with the requirements for pancreatic islet isolation from adult tissue, preparation of the rodent foetal pancreas for transplantation is less labour intensive. In addition, the foetal tissue probably provides a much higher yield of potentially functional endocrine tissue. With adult pancreas, the need to separate the mature islets from the exocrine portion of the gland leads to a low islet yield, but this step is not necessary with rodent foetal pancreas, as the organ can be transplanted *en bloc*. Mullen and her colleagues (1977) have shown that under ideal conditions a single foetal pancreas will completely reverse streptozotocin-diabetes in the rat, and Simeonovic (personal communication), has used a single isogeneic foetal pancreas

to reverse diabetes in the mouse. Similar results have been achieved using isolated rat or mouse islets, but several donors have been required (Gray & Watkins 1976; Nash *et al* 1978; Lacy *et al* 1979; Chapter 4 of this thesis). The above-mentioned advantages imply that human foetal pancreas may be the better choice for use in clinical transplantation, but application of the technique of organ culture to experimental foetal pancreas allotransplantation in rodents has so far been unsuccessful. Mandel & Higginbotham (1979) and (Garvey *et al* 1980) cultured foetal tissue in a 5% CO_2 and air gas phase, and failed to show any long-term beneficial effect of organ culture, while Chase, Ocrant & Talmage (1979) found that rat foetal pancreas became necrotic when cultured on grids in a 95% O_2/5% CO_2 gas phase.

The aim of this present study was to determine whether there is an intrinsic difference in immunogenicity between foetal mouse pancreas and adult mouse pancreatic islets. To show this, foetal mouse pancreas and adult islets from the same inbred strain of mouse were cultured under similar conditions prior to allotransplantation.

RESULTS

Survival of cultured and uncultured islet allografts

Aggregates of uncultured BALB/c(H-2^d) islets were transferred to the kidney capsule of normal CBA(H-2^k) recipient mice.

Groups of 4 allografted animals were killed 14 and 28 days after transplantation, and the allografts examined histologically (Table 7.1).

TABLE 7.1 Survival of cultured and uncultured islet allografts

Time in organ culture	Time after transplantation (days)	Histological score	
		Mononuclear cell infiltration	Integrity of islets
None	14	+	++++
		+++	+++
		++++	+
		++	++++
7 days	14	0	+++++
		+	+++++
		0	+++++
		0	+++++
None	28	+++	++++
		++++	++
		++++	+
		++++	+
7 days	28	0	+++++
		0	+++++
		0	+++++
		0	+++++

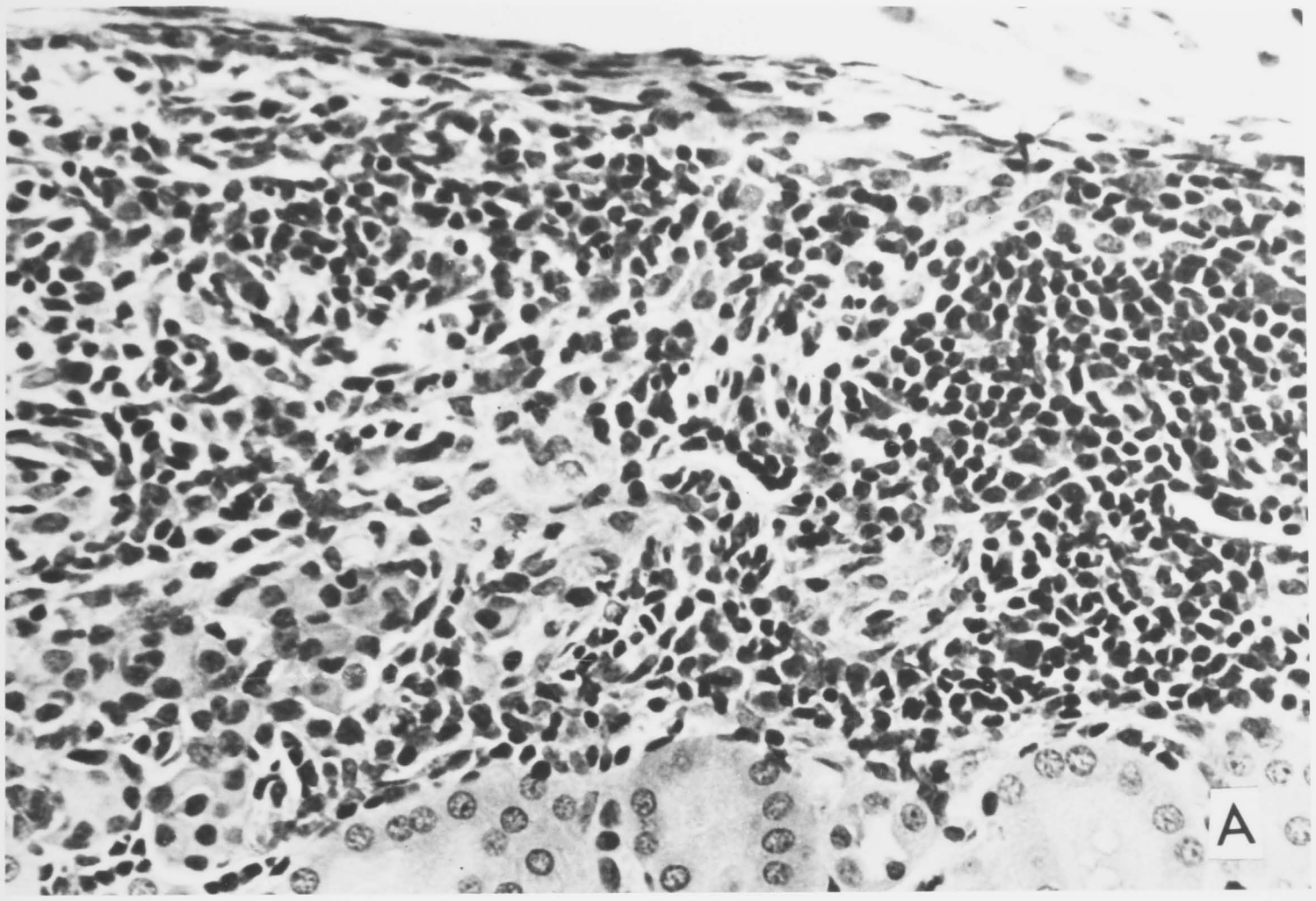
At 14 days after transplantation, there was a mononuclear cell response to the transplanted tissue, but beta cells staining with aldehyde-fuchsin were still present at this time. By 28 days the allografted tissue was largely rejected (Figure 7.1A).

In contrast to these findings, allografts of islet tissue cultured for 7 days in 95% O_2 and 5% CO_2 before grafting showed no evidence of rejection, with all 5 allografts surviving completely intact for 84 days after transplantation. The allografted tissue was well vascularised, and beta cells in the transplant stained strongly with aldehyde-fuchsin (Figure 7.1B).

Organ culture of BALB/c foetal pancreas

At 16 to 17 days of gestation, the foetal mouse pancreas consists of a small number of immature islets, some duct tissue, and a considerable amount of acinar tissue at various stages of differentiation. In addition, careful examination of histological sections showed the presence of primitive lymphoid tissue associated with the foetal pancreas (Figure 7.2).

After a period of 10 days in organ culture, the foetal pancreas condensed into a compact mass which appeared to be encapsulated by a thin film of connective tissue. On histological examination, large well-differentiated islets as well as numerous acini, were identified, particularly



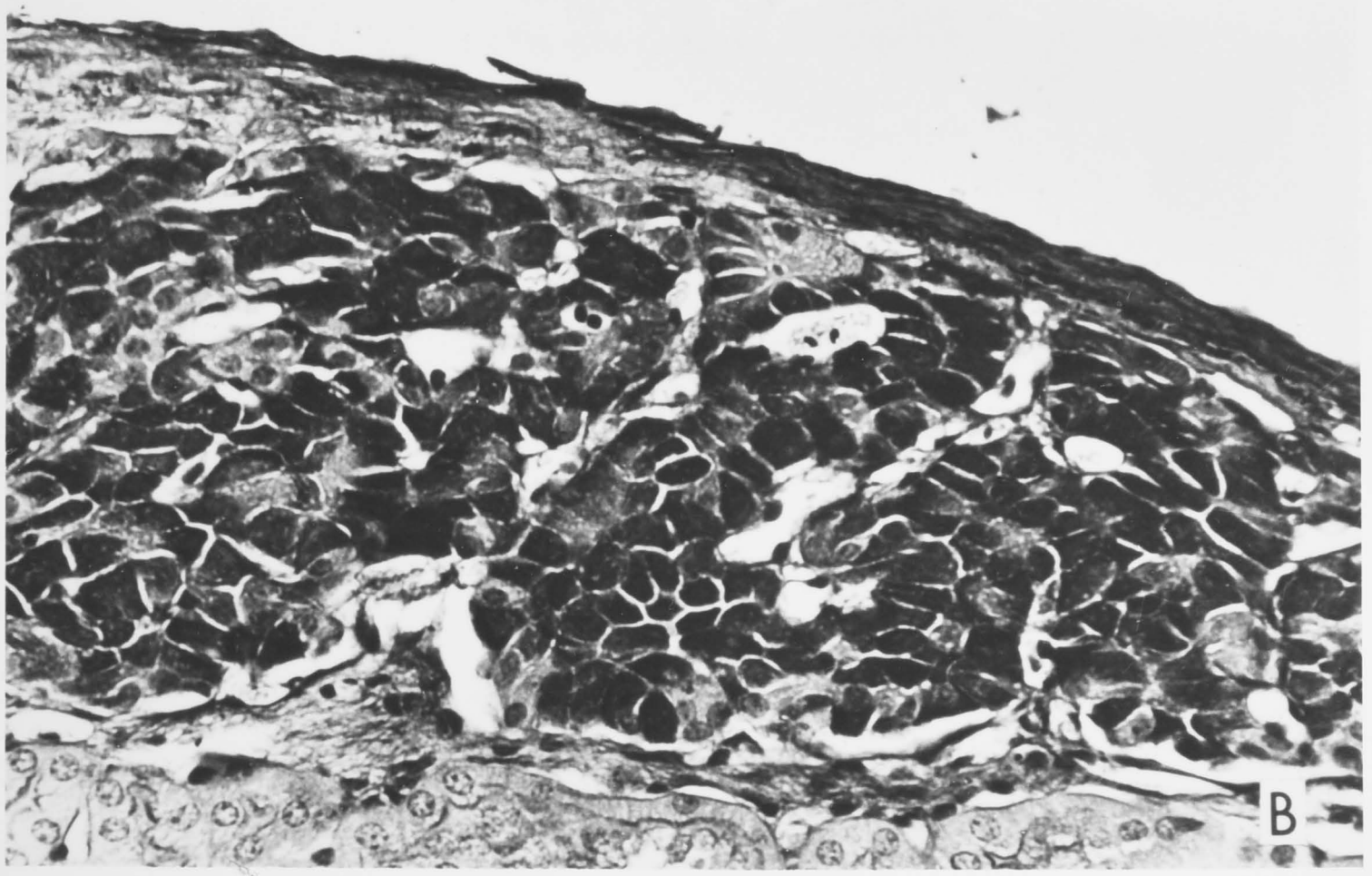


Figure 7.1(A) The allograft response to BALB/c isolated adult islets 28 days after transplantation under the kidney capsule of a normal CBA recipient mouse. The graft is almost completely rejected (haematoxylin and eosin x 1370)

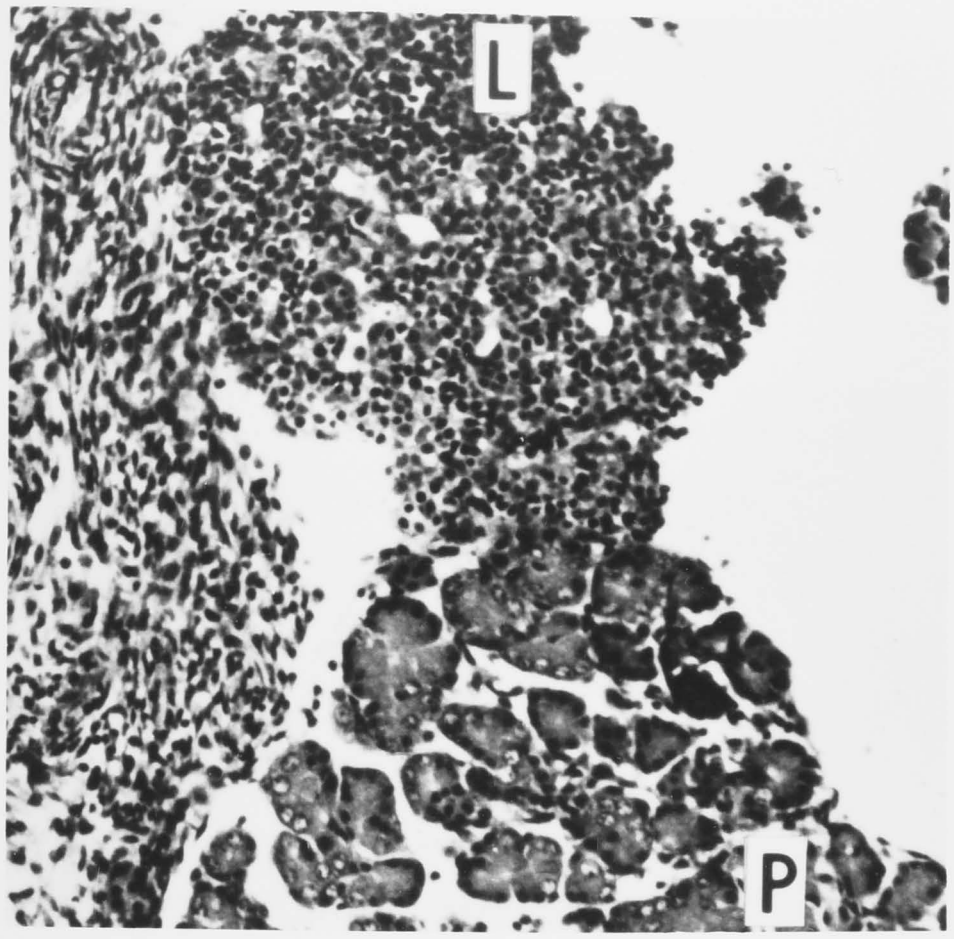
Figure 7.1(B) An allograft of 7-day cultured BALB/c adult islets 84 days after transplantation under the kidney capsule of a normal CBA recipient mouse. Note the darkly stained beta cells within the transplanted tissue, and the lack of any allograft response. (aldehyde-fuchsin x 1370)

in the peripheral regions of the tissue (Figure 7.3). The relative proportion of islet tissue in the explant had increased, and lymphoid elements were less obvious. A few scattered lymphoid cells were seen in some sections of 10 day cultured foetal pancreas. The central portion of the cultured tissue usually showed some degree of necrosis. After 17 days of organ culture, the acinar tissue had completely degenerated, and all that remained were well formed islets, duct tissue, and connective tissue.

Isotransplantation of uncultured and cultured foetal pancreas

When BALB/c foetal pancreas (16 to 17 days gestation) was transplanted to the kidney capsule of syngeneic recipients, no evidence of rejection was seen. The tissue was rapidly revascularised, and 28 days after transplantation it consisted primarily of differentiated islets, some duct tissue, and a massive accumulation of fat. No acinar tissue was present, but a surprising finding was the presence of a large mass of lymphoid tissue in 3 of the 4 isografts examined (Figure 7.4). This tissue, which was structurally similar to a lymph node, presumably resulted from the continued development of the lymphoid tissue seen in association with the freshly isolated foetal pancreas.

Isografts of 10-day cultured foetal mouse pancreas were rapidly revascularised. At 28 days after transplantation, these grafts were characterised by the presence of large



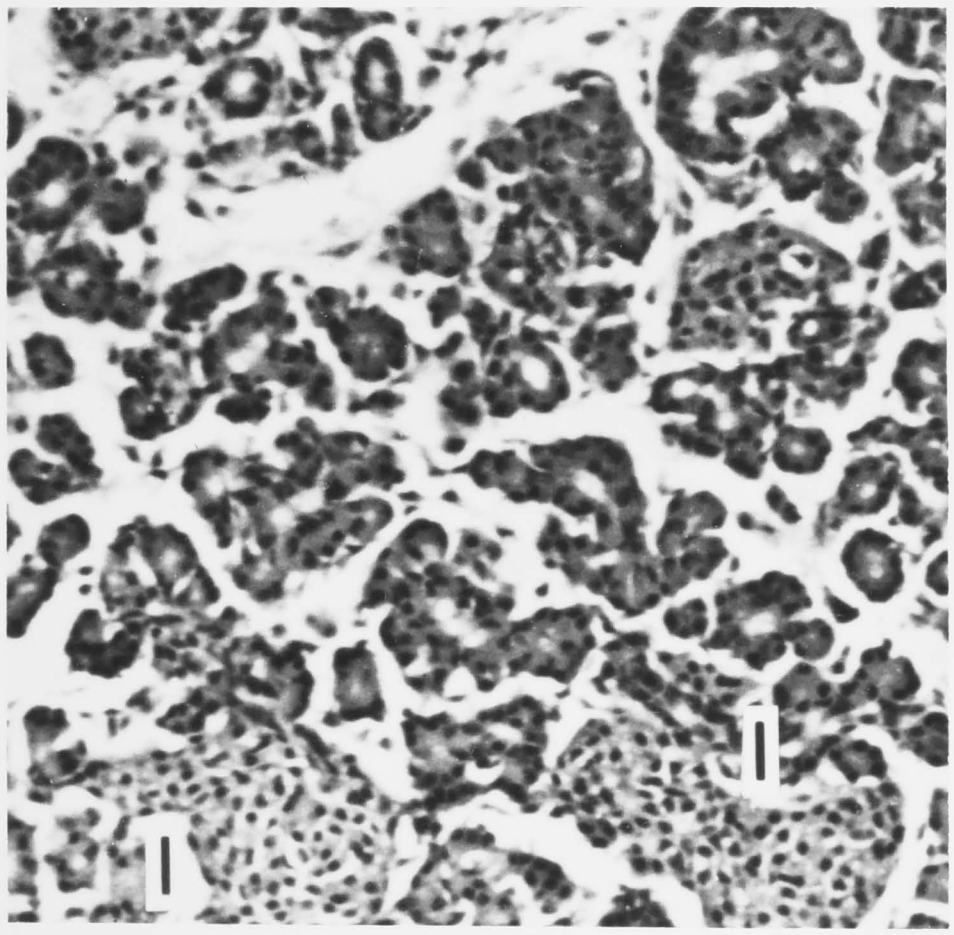


Figure 7.2(Top) The histological appearance of BALB/c foetal mouse pancreas (16 to 17 days of gestation) with primitive lymphoid tissue (L) adjacent to acinar tissue (P) (haematoxylin and eosin x 550)

Figure 7.3(Bottom) Histological section of BALB/c foetal mouse pancreas (16 to 17 days of gestation) cultured for 10 days in a 95% O_2 and 5% CO_2 gas phase. Discrete islets (I) and well differentiated acinar tissue are present. (haematoxylin and eosin x 550)

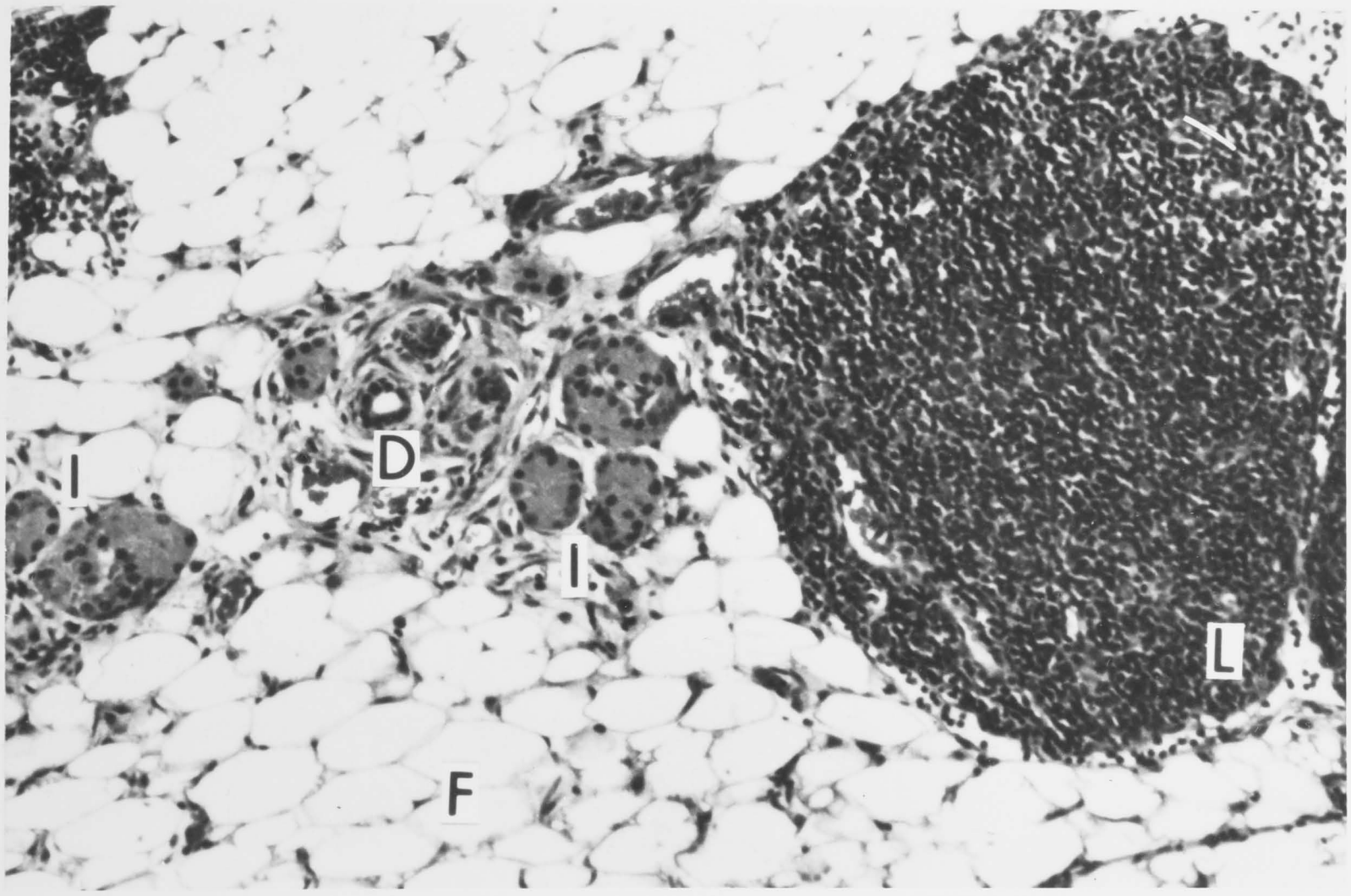


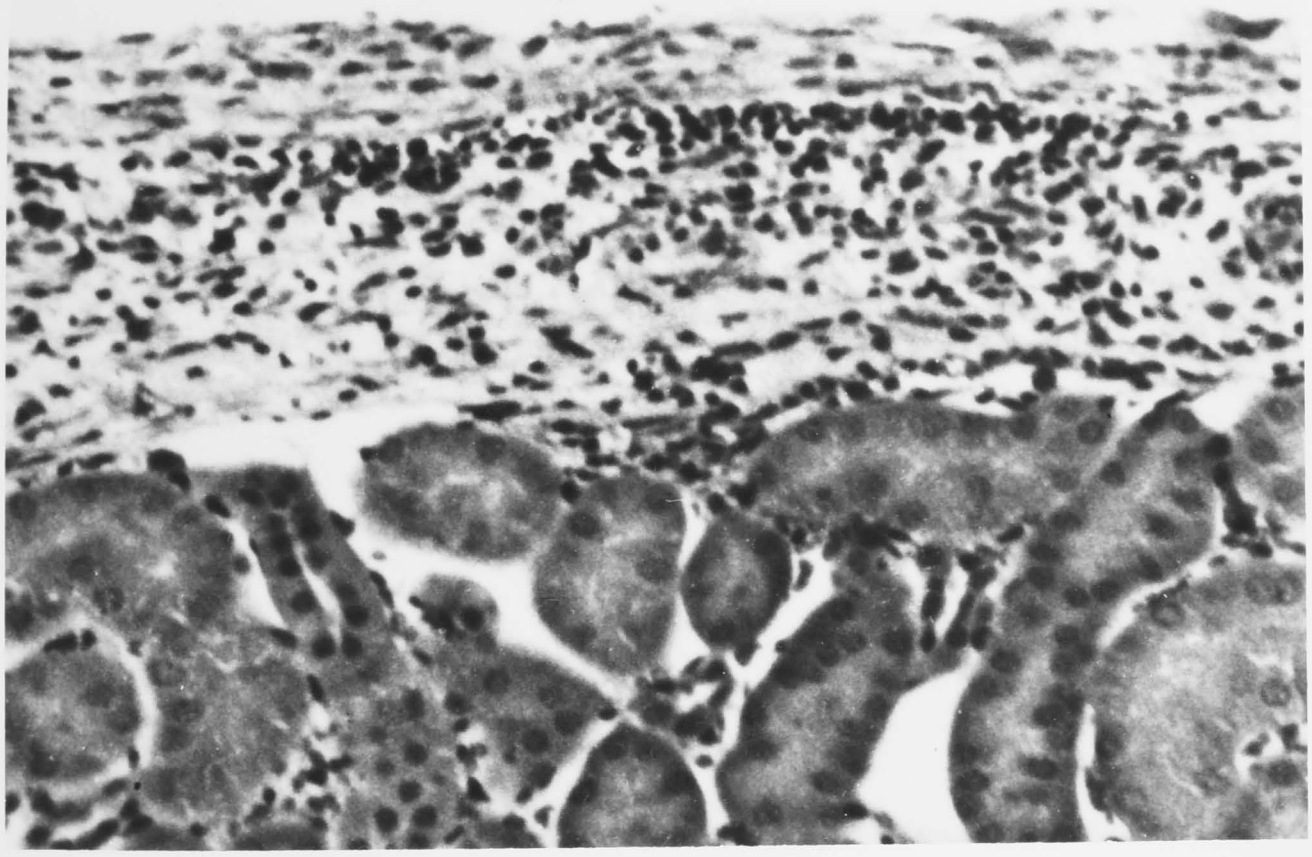
Figure 7.4 Isograft of BALB/c foetal mouse pancreas 28 days after transplantation under the kidney capsule. Note presence of islets (I) duct tissue (D), adipose tissue (F) and a mass of lymphoid tissue (L). (haematoxylin and eosin x 550)

intact islets, duct tissue, some fibrotic tissue, and fat deposition. However, no lymph-node development was observed after transplantation of the cultured tissue.

Allotransplantation of cultured and uncultured foetal pancreas

Allografts of foetal mouse pancreas were acutely rejected; within 14 days of transplantation all that remained was scar tissue and mononuclear cells at the site of transplantation (Figure 7.5). Allografts of 10-day cultured foetal pancreas were also rejected, but not quite as acutely as the uncultured tissue. By 14 days post transplantation, the tissue showed signs of rejection, but some damaged islets could still be seen (Figure 7.6). These grafts were completely rejected 28 days after transplantation; histological examination showed the presence of scar tissue, mononuclear cell infiltration, and no trace of pancreatic tissue.

In contrast to these findings, 3 out of 4 allografts of 17-day cultured foetal pancreas segments showed no sign of rejection 28 days after transplantation. On macroscopic examination, these grafts appeared well vascularised, and histological examination showed that the beta cells of the foetal islets stained quite densely with aldehyde-fuchsin (Figure 7.7), and resembled cultured isografts that had been examined 28 days after transplantation (Figure 7.8). The remaining graft consisted only of fibrotic tissue. It is possible that this allograft was rejected; alternatively, the foetal pancreatic



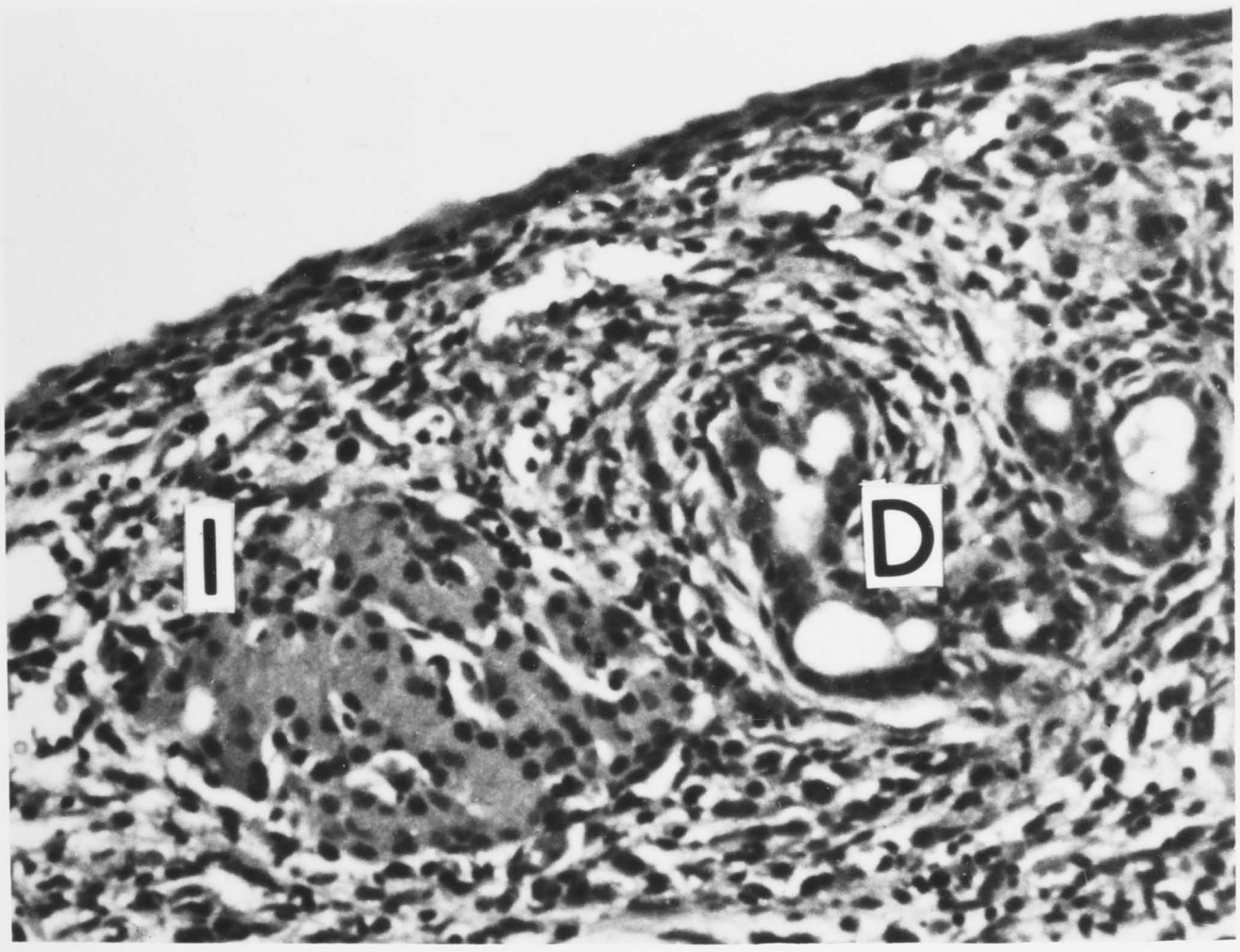
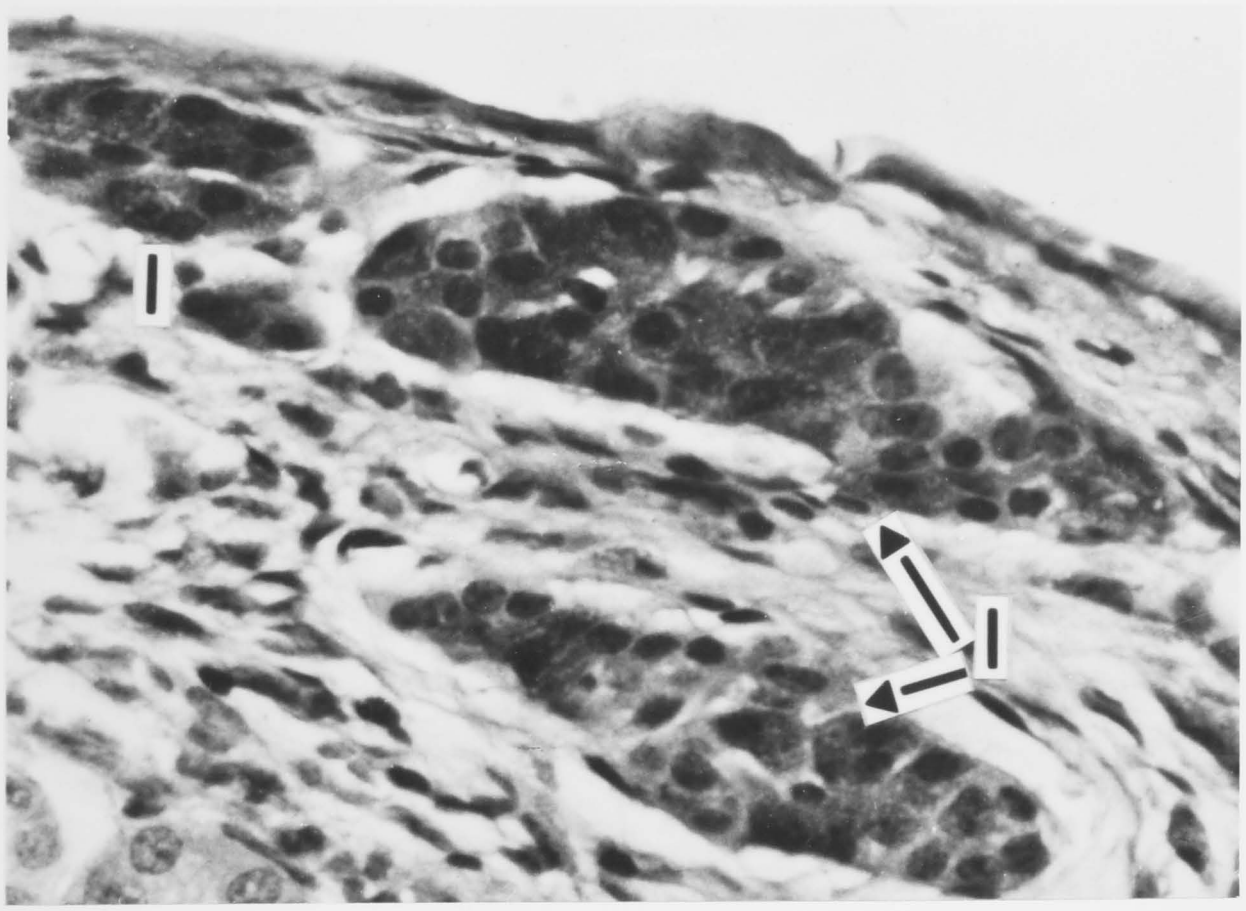


Figure 7.5(Top) Allograft of uncultured BALB/c foetal pancreas 14 days after transplantation under the kidney capsule of a CBA recipient. Note scar tissue and mononuclear cell infiltration (haematoxylin and eosin x 900)

Figure 7.6(Bottom) Allograft of a 10-day cultured BALB/c foetal pancreas 14 days after transplantation to the kidney capsule of a normal CBA recipient. Note the mononuclear cell infiltration. Islet tissue (I) and duct tissue (D) is present. (haematoxylin and eosin x 900)



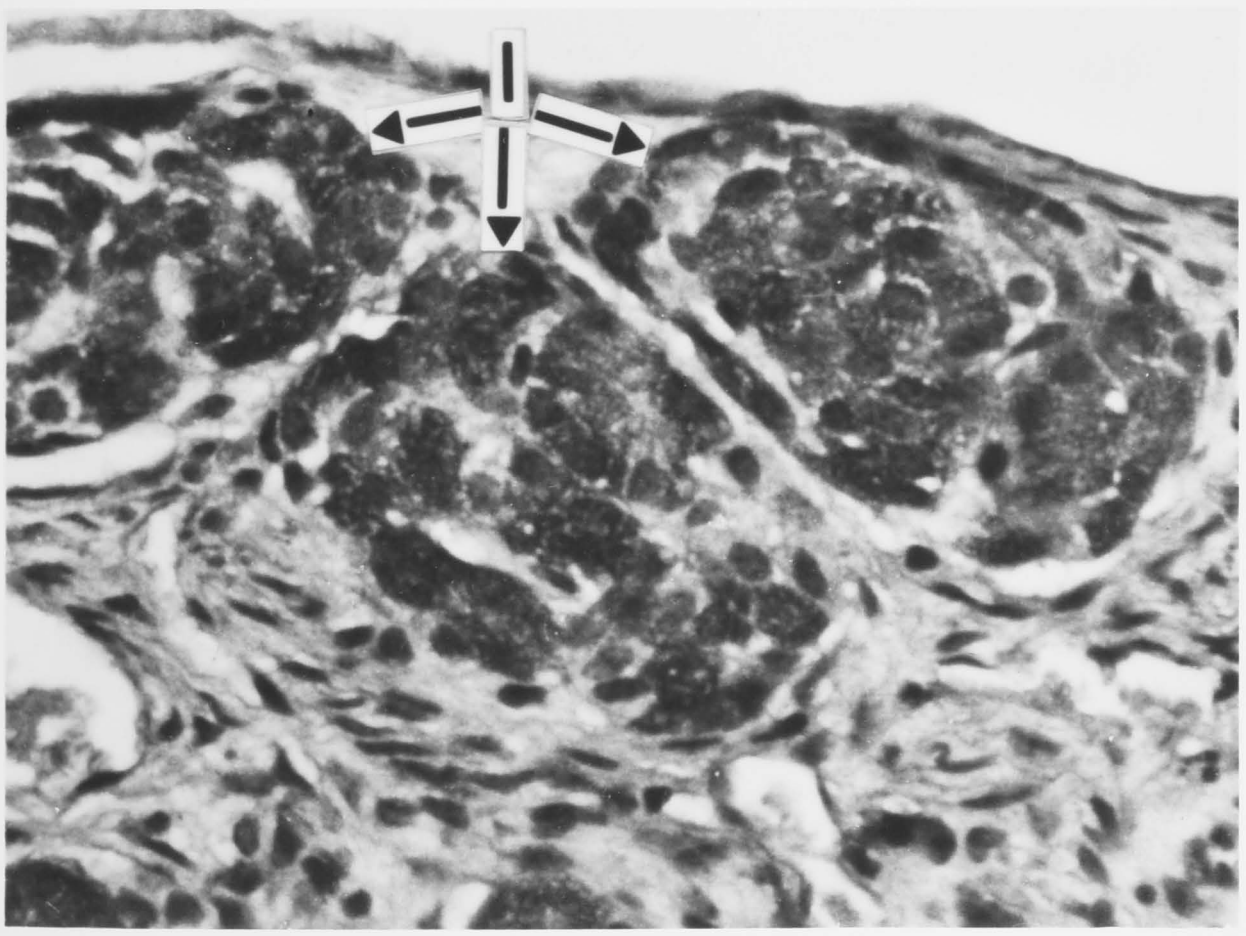


Figure 7.7(Top) Allograft of 17-day cultured BALB/c foetal pancreas 28 days after transplantation under the kidney capsule of a CBA recipient mouse. Note the lack of an allograft response, and the presence of intact islets (I) containing granulated beta cells. (aldehyde fuchsin x 1370)

Figure 7.8(Bottom) Isograft of 10-day cultured BALB/c foetal pancreas 28 days after transplantation under the kidney capsule of a BALB/c recipient. Note development of islets (I) containing granulated beta cells. (aldehyde fuchsin x 1370)

segment may have failed to survive the period of culture prior to transplantation. There was no evidence of mononuclear cell infiltration at the graft site, and this suggests the possibility of a technical failure.

DISCUSSION

Adult mouse pancreatic islets were found to be more readily conditioned for allotransplantation by organ culture than was the foetal mouse pancreas. This difference is likely to be due to the large collections of lymphoid tissue that were found to be associated with the BALB/c foetal pancreas. This rather diffuse gland is intimately associated with mesentery, and since collections of lymphocytes are not seen within the substance of the foetal pancreas, the lymphoid tissue probably represents developing mesenteric lymph nodes. Mandel (personal communication) has not observed lymphoid collections with isografts of CBA foetal pancreas; the explanation for this apparent strain difference has not yet been determined, but nevertheless, the presence of donor-strain lymphoid tissue at transplantation would explain why tissue similar to lymph nodes was found in isografts of uncultured foetal pancreas. Organ culture before transplantation appears to damage this lymphoid tissue, as discrete lymph nodes do not develop after isotransplantation of the cultured foetal pancreas. However, lymphoid tissue remnants have been seen in association with the 10-day cultured foetal

pancreas, and it is possible that this residual lymphoid tissue triggers the rejection process. Nevertheless, it is possible to successfully transplant BALB/c foetal mouse pancreas to non-immunosuppressed CBA recipients if a 17 day culture period is used for tissue conditioning. These findings show that the optimal conditions for culture vary according to the type of tissue being examined, as it is clear that the culture conditions required to prepare foetal mouse tissue for allotransplantation are more stringent than those required to facilitate adult mouse islet transplantation.

CHAPTER EIGHT

GENERAL DISCUSSION

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GENERAL DISCUSSION

In this thesis, the primary aim was to find out whether an organ culture technique previously shown to abolish the immunogenicity of mouse thyroid tissue (Lafferty *et al* 1975) could be adapted to produce the same results with pancreatic islets. These have gained a notorious reputation for being highly immunogenic (Reckard & Barker 1973; Nash, Peters & Bell 1977; Frangipane, Barker & Silvers 1977; Garvey *et al* 1979), but it is clear from the data presented in Chapter 3 of this thesis that organ culture for 7 days in a 95% oxygen gas phase can eliminate mouse pancreatic islet immunogenicity at the time of allografting across major histocompatibility barriers. In Chapter 5 it was shown that this effect was due to a loss of allogeneic stimulatory capacity by the cultured tissue. Cultured islet allografts will also reverse experimental diabetes, as shown in Chapter 4, and it is reasonable to hope that organ culture will have similar beneficial effects on human islet tissue being prepared for allotransplantation to insulin-dependent diabetics. If this does prove to be the

case, and human islet allografts conditioned by organ culture are not rejected by their hosts, it would be fair to say that a curative treatment for insulin-dependent diabetes was at hand. Unfortunately, the reality of the present situation is somewhat less encouraging. Isolation of large numbers of islets from the adult human pancreas is extremely difficult, due to the fibrous and compact nature of the organ (Scharp *et al* 1980) and until a method producing high yields of islets is found, it is unlikely that allo-transplantation of cultured isolated human islets will be feasible. Nevertheless, Sutherland *et al* (1980) have shown that autografts of collagenase-digested human pancreatic fragments can maintain glucose homeostasis. The use of this form of islet preparation as an allograft usually results in failure of the graft to function, presumably as a result of graft rejection (Sutherland *et al* 1980; Largiader, Kolb & Binswanger 1980) although Largiader's group has described one patient whose allograft functioned long term, and enabled insulin therapy to be withdrawn. Whether organ culture of human pancreatic fragments under conditions of high oxygen tension prior to allotransplantation will reduce or eliminate their immunogenicity remains to be shown, but such an approach is worth attempting in view of the favourable findings with cultured islet allografts documented in this thesis.

Lacy, Davie & Finke (1979b) have shown that organ culture and ALS therapy have a synergistic effect on the prolongation

of survival of rat islet allografts. Used alone, ALS has proven to be the most effective immunosuppressive agent in experimental islet allograft studies (Barker, Naji & Silvers 1980), and it may eventuate that a combination of donor tissue pretreatment and ALS or antilymphocyte globulin (ALG) therapy will prove to be effective in human islet allotransplantation. Newer immunosuppressive agents such as cyclosporin also need to be tried in combination with organ culture techniques, even though the drug has not been shown to be particularly effective in significantly prolonging murine islet allograft survival when used alone (Bell *et al* 1980; Garvey *et al* 1980b; Rynasiewicz *et al* 1980).

The use of human foetal pancreas offers a way around the logistical problems of islet isolation. It has already been shown by a number of workers that murine foetal pancreas isografts will reverse experimental diabetes (Brown *et al* 1976; Mandel & Higginbotham 1979; Simeonovic - personal communication), and in Chapter 7 of this thesis, evidence is presented showing that foetal mouse pancreas cultured in a 95% oxygen gas phase for 17 days prior to transplantation fails to induce an allograft response 28 days following grafting across a major histocompatibility barrier. In contrast, uncultured allografts had always rejected by 14 days after transplantation. The results with cultured foetal murine pancreas point to the exciting possibility of using organ culture to modulate the immunogenicity of

human foetal tissue. Maitland, Parry & Turtle (1980) and Agren *et al* (1980) have shown that human foetal endocrine pancreas will survive and function in organ culture for at least 14 days when cultured in a gas phase of air, and since cultured foetal pancreas will reverse experimental diabetes in mice when grafted to isogeneic recipients after culture in 5% CO_2 and air (Mandel & Higginbotham 1979), or in 95% O_2 and 5% CO_2 (Simeonovic - personal communication), it should be possible to apply the high oxygen tension culturing technique to condition human foetal pancreas prior to transplantation.

Transplantation of segmental human pancreas with vascular anastomosis, HLA-DR matching of donor and recipient, (Ting & Morris 1980), and host immunosuppression is also likely to resume greater clinical importance if cyclosporin continues to produce favourable results in experimental and clinical pancreas allotransplantation (Calne 1980; Rynasiewicz *et al* 1980; McMaster *et al* 1980; Garvey *et al* 1980b) and the new surgical technique of pancreatic duct drainage into the peritoneal cavity reduces the incidence of surgical complications (Kyriakides, Nuttal & Miller 1979).

To conclude, it is evident that organ culture of endocrine pancreas prior to allotransplantation is just one of several approaches now available that offer a potential solution to the problem of allograft rejection. Accordingly, it is likely that cure of insulin-dependent diabetes by endocrine pancreas allotransplantation will become a reality within the next few years.

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